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DESCRIPTION

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## THREE-DIMENSIONAL TISSUE STRUCTURE

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## TECHNICAL FIELD

The present invention relates to a three-dimensional structure applicable to the heart, and more particularly, to a three-dimensional structure applicable to the heart, which comprises cells derived from parts of an adult other than the heart. The present invention also relates to a method for producing such a three-dimensional structure.

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## BACKGROUND ART

Myocardial infarct is an irreversible injury (Ho K.K., Anderson K.M., Kannel W.B., Grossman W., Levy D., Circulation, 1993; 88: 107-115). Ischemic heart diseases are the cause of death responsible for 50% of all cardiovascular system-related deaths and the major cause of congestive heart failure. The 1-year mortality observed in patients who are diagnosed as having congestive heart failure and eventually die from chronic heart disease is 20% (American Heart Association, Dallas, Tex: American Heart Association; 2001). Most therapies currently available to clinicians can significantly improve the prognosis of patients suffering from acute myocardial infarct. Angioplasty and thrombolytic agents may remove the cause of the acute myocardial infarct, though the period of time from the onset of occlusion to reperfusion determines the degree of irreversible myocardial injury (Ryan T.J., Antman E.M., Brooks N.H., Califf R.M., Hillis L.D., Hiratzka L.F., Rapaport E., Riegel B., Russell R.O., Smith E.E. III, Weaver

W.D., Gibbons R.J., Alpert J.S., Eagle K.A., Gardner T.J., Garson A. Jr., Gregoratos G., Ryan T.J., Smith S.C. Jr., J. Am. Coll. Cardiol., 1999; 34: 890-911). No clinically used pharmaceutical agent or treatment has an efficacy on  
5 the replacement of myocardial scars with functional contraction tissue. There is a demand for a novel therapy for regenerating normal cardiomyocytes.

Cardiomyoplasty has been proposed as a surgical  
10 method for improving the function of the left ventricle (LV) of a patient suffering from congestive heart failure, however, the effect thereof on the cardiac function remains unclear (Corin W.J., George D.T., Sink J.D. et al., J. Thorac. Cardiovasc. Surg., 1992, 104:1662-1671; Kratz J.M., Johnson  
15 W.S., Mukherjee R. et al., J. Thorac. Cardiovasc. Surg., 1994, 107:868-878; Carpentier A., Chachques J.C., Lancet, 1985, 8840:1267; and Hagege A.A., Desnos M., Chachques J.C. et al., Preliminary report: follow-up after dynamic cardiomyoplasty, Lancet, 1990, 335:1122-1124). Recently,  
20 implantation of a biologically modified heart graft, in which a biodegradable scaffold is used, has been proposed as another novel approach. However, the graft hardly attaches to the myocardium, resulting in the least possible benefit for the improvement of the cardiac function (Leor J., Etzion S.A.,  
25 Dar A. et al., Circulation, 2000; 102 [suppl. III] III-56-III-61; and Li R.K., Jia Z.Q., Weisel R.D. et al., Circulation, 1999; 100 [suppl II]: II-63-II-69). The histological and electrical integration of biologically modified heart tissue and a recipient heart may be crucial  
30 for the regeneration of impaired myocardium.

The recent development of tissue engineering is expected to make possible the production of a functional

heart tissue using a novel technique, in which cell sheets are three-dimensionally layered without any biodegradable substitute for extracellular matrices (ECM) (Okano T., Yamada N., Sakai H., Sakurai Y., J. Biomed. Mater. Res., 1993; 27:1243-1251). In this novel technique, both intracellular adhesion and adhesion proteins within a confluent cultured cell monolayer are fully maintained. Endogenous ECM supporting a cell sheet whose base portion has been collected by a collecting method (Kushida A., Yamato M., Konno C., Kikuchi A., Sakurai Y., Okano T., J. Biomed. Mater. Res., 45:355-362, 1999) plays an important role as an adhesion factor for the integration to the recipient heart. Further, the cardiomyocyte sheet is a pulsating 3-D heart construct which transmits electricity (Shimizu T., Yamato M., Akutsu T. et al., Circ. Res., Feb 22, 2002, 90(3):e40). However, it is unknown whether or not cardiomyocyte sheets retain their functions after *in vivo* implantation.

The recent progress in tissue engineering has the potential of providing an implantable functional tissue comprising various cells and an extracellular matrix.

Implantation of organs (e.g., heart, blood vessel, etc.) using an exogenous tissue is mainly hindered by immunological rejections. Changes occurring in allografts and xenografts were first described 90 or more years ago (Carrel A., 1907, J. Exp. Med. 9:226-228; Carrel A., 1912, J. Exp. Med. 9:389-392; Calne R.Y., 1970, Transplant Proc. 2:550; and Auchincloss 1988, Transplantation 46:1). Rejection to artery grafts pathologically leads either to enlargement (up to rupture) or occlusion of the grafts. The former is caused by decomposition of extracellular matrices, while the latter is caused by proliferation of cells in a

blood vessel (Uretsky B.F., Mulari S., Reddy S., et al., 1987, Circulation 76:827-834). Such grafts are often made from non-biological materials which lead to adverse effects.

5                   Recently, cell implantation has attracted attention as a therapy utilizing biological material. However, the implantation of human myoblasts into the infarcted heart has the following drawbacks: 1. damage and loss of  
10                   implantation cells; 2. tissue injury of the recipient heart during implantation; 3. tissue supply efficiency to the recipient heart; 4. occurrence of arrhythmia; 5. difficulty in treating the entirety of the infarcted site; and the like. Therefore, cell implantation cannot be said to be very successful.

15                   Myocardium-derived sheets have been developed. Typically, autologous myocardium is required for the myocardium-derived sheet in view of immune reactions. Therefore, the applications of the sheet are limited.

20                   Accordingly, there is a keen demand for a prosthetic tissue, a three-dimensional structure, or a sheet capable of withstanding implantation operations, being used in actual operations, and being produced by culture.

25                   

### DISCLOSURE OF THE INVENTION

                  An object of the present invention is to provide a prosthetic tissue or sheet capable of withstanding  
30                   implantation operations, being used in actual operations, and being produced by culture. Another object of the present invention is to provide a novel therapy, which is an alternative to cell therapy. The present invention is



particularly directed to production of a prosthetic tissue comprising cells derived from parts other than the myocardium, which can withstand implantation operations.

5           The above-described objects of the present invention were achieved by providing a three-dimensional structure comprising cells derived from parts other than the myocardium. The objects of the present invention were partially achieved by finding that by culturing cells under specific culture  
10 conditions, the cells are unexpectedly organized into a tissue, and the resultant prosthetic tissue is capable of being detached from culture dishes.

          The present invention was also achieved by  
15 unexpectedly finding that a three-dimensional structure comprising cells derived from parts other than the myocardium can function in a manner similar to that of the myocardium.

          Therefore, the present invention provides the  
20 following.

(Non-cardiac sheet/three-dimensional structure)

(1) A three-dimensional structure applicable to  
25 heart, comprising a cell derived from a part other than myocardium of an adult.

(2) A structure according to item 1, wherein the cell  
is a stem cell or a differentiated cell.

30 (3) A structure according to item 1, wherein the cell is a mesenchymal cell.

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(4) A structure according to item 1, wherein the cell is derived from a myoblast.

5 (5) A structure according to item 4, wherein the myoblast is a skeletal myoblast.

(6) A structure according to item 1, wherein the cell is a fibroblast.

10 (7) A structure according to item 1, wherein the cell is a synovial cell.

(8) A structure according to item 1, wherein the cell is derived from a stem cell.

15 (9) A structure according to item 1, wherein the cell is derived from a subject, the structure being applied to the subject.

20 (10) A structure according to item 1, wherein the cell is not derived from a subject, the structure being applied to the subject.

25 (11) A structure according to item 1, wherein the structure expresses at least one non-adult heart marker selected from the group consisting of myosin heavy chain IIa, myosin heavy chain IIb, myosin heavy chain IIc(IIx), CD56, MyoD, Myf5, and myogenin.

30 (12) A structure according to item 11, wherein an expression level of the non-adult heart marker in the structure is at least 50% of an expression level of the non-adult heart marker in skeletal myoblasts.

- 7 -

(13) A structure according to item 1, wherein the three-dimensional structure expresses all of myosin heavy chain IIa, myosin heavy chain IIb, myosin heavy chain IID(IIx),  
5 CD56, MyoD, Myf5, and myogenin.

(14) A structure according to item 13, wherein an expression level of each of myosin heavy chain IIa, myosin heavy chain IIb, myosin heavy chain IID(IIx), CD56, MyoD,  
10 Myf5, and myogenin in the structure is at least about 50% of an expression level thereof in skeletal myoblasts.

(15) A structure according to item 13, wherein an expression level of each of myosin heavy chain IIa, myosin heavy chain IIb, myosin heavy chain IID(IIx), CD56, MyoD,  
15 Myf5, and myogenin in the structure is at least about 100% of an expression level thereof in skeletal myoblasts.

(16) A structure according to item 1, wherein the  
20 cell derived from a part other than myocardium is a cell not derived from heart.

(17) A structure according to item 1, wherein the applicability to heart includes applicability to myocardium.  
25

(18) A structure according to item 1, comprising a monolayer cell sheet.

(19) A structure according to item 1, comprising a  
30 multilayer cell sheet.

(20) A structure according to item 19, wherein the multilayer cell sheet has biological connection.

(21) A structure according to item 20, wherein the biological connection is selected from the group consisting of connection via extracellular matrix, electrical connection, and connection without scaffold.

(22) A medicament, comprising a three-dimensional structure according to any one of items 1 to 21.

(23) A medicament according to item 22, wherein the heart has a disease or disorder selected from the group consisting of heart failure, ischemic heart disease, myocardial infarct, cardiomyopathy, myocarditis, hypertrophic cardiomyopathy, dilated phase hypertrophic cardiomyopathy, and dilated cardiomyopathy.

(24) A method for producing a three-dimensional structure applicable to heart comprising a cell derived from a part other than myocardium of an adult, the method comprising the steps of:

a) culturing the cell derived from the part other than myocardium of an adult on a cell culture support grafted with a temperature responsive macromolecule having an upper limit critical solution temperature or lower limit critical solution temperature to water of from 0°C to 80°C;

b) setting a culture medium temperature to the upper limit critical solution temperature or more or the lower limit critical solution temperature or less; and

c) detaching the cultured cell as a three-dimensional structure.

(25) A method according to item 24, wherein a treatment using a protein degrading enzyme is not performed

in or before the detaching step.

(26) A method according to item 24, wherein the  
temperature responsive macromolecule is  
5 poly(N-isopropylacrylamide).

Hereinafter, the present invention will be described  
by way of preferred embodiments. It will be understood by  
those skilled in the art that the embodiments of the present  
10 invention can be appropriately made or carried out based  
on the description of the present specification and commonly  
used techniques well known in the art. The function and  
effect of the present invention can be easily recognized  
by those skilled in the art.

15

The present invention provides an implantable  
prosthetic tissue. This tissue has a large size, which cannot  
be achieved by conventional techniques, and has an excellent  
strength. Thereby, it is made possible to treat sites which  
20 cannot be conventionally accessible to implantation  
treatment using conventional prosthetic materials. The  
present invention makes it possible to provide a prosthetic  
tissue or three-dimensional structure made of not only  
myocardial tissue but also parts other than the myocardium.  
25 Therefore, parts other than autologous myocardium can be  
used as material to conduct implantation therapies.

#### BRIEF DESCRIPTION OF THE DRAWINGS

30 Figure 1A shows an example in which a prosthetic  
tissue of the present invention is produced using a  
temperature responsive polymer.

Figure 1B shows another example in which a prosthetic tissue of the present invention is produced using a temperature responsive polymer.

5           Figure 2 shows an example in which a therapy using a prosthetic tissue of the present invention is compared with a cell therapy.

10           Figure 3 shows an exemplary therapy scheme using a prosthetic tissue of the present invention.

15           Figure 4 shows a limit of myocardial regenerative therapies using cell implantation. As shown in the right portion, an injured site is not completely healed by cells in cell implantation.

            Figure 5 shows a limit of tissue implantation using a scaffold.

20           Figure 6 shows an example of implantation of a prosthetic tissue of the present invention into an infarcted heart.

25           Figure 7 shows a state of a prosthetic tissue of the present invention after implantation. The left portion shows the tissue 2 weeks after implantation, while the right portion shows the tissue 8 weeks after implantation. HE staining is shown over the left panel (left:  $\times 100$ ; right:  $\times 200$ ). Factor VIII staining is shown below the left panel, 30 while Connexin 43 staining is shown to the right. The right panel shows HE staining (upper:  $\times 40$ ; lower:  $\times 100$ ).

            Figure 8 is an ultrasound echogram showing an example

of evaluation of cardiac function ameliorated by a prosthetic tissue of the present invention. The left portion shows a control, while the right portion shows a cardiomyocyte sheet.

5               Figure 9 shows an example of evaluation of cardiac function ameliorated by a prosthetic tissue of the present invention. In the figure, the ejection fraction (EF), the fractional shortening (FS), and the endo-systolic area (ESA) are shown. Squares indicate the cardiomyocyte sheet, while  
10              triangles indicate the control. A left panel photograph is an ultrasound echo photograph (upper: control; lower: cardiomyocyte sheet).

              Figure 10 shows a technique for  
15              electrophysiological evaluation of a prosthetic tissue of the present invention. The right portion schematically shows changes in electric potential, while the right portion shows numerical values of threshold. The right portion shows a control, and a fibroblast sheet of the present invention  
20              and a cardiomyocyte sheet of the present invention.

              Figure 11 shows electrophysiological evaluation of a prosthetic tissue of the present invention. The upper left portion shows a normal heart, the lower left portion shows  
25              an infarct model, and the lower right portion shows a therapy using a cardiomyocyte sheet.

              Figure 12 shows an example of procedures for isolating and culturing myoblasts in a method according to  
30              the present invention.

              Figure 13 shows an example of prosthetic tissue culture containing myoblasts in a method according to the

present invention.

Figure 14 shows an exemplary experimental scheme using a prosthetic myoblast tissue of the present invention.

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Figure 15 shows photographs indicating a myoblast prosthetic tissue of the present invention 4 weeks after implantation ( $\times 10$ ,  $\times 200$ , and  $\times 1000$ ).

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Figure 16 shows an exemplary implantation operation using a prosthetic myoblast tissue of the present invention.

Figure 17 shows an exemplary implantation of a prosthetic myoblast tissue of the present invention (histological staining). The upper portion shows the prosthetic tissue, cell injection, and control from the right ( $\times 10$ ). The lower portion shows a photograph ( $\times 400$ ) for each.

15

Figure 18 shows an ultrasound echogram of implantation of a prosthetic myoblast tissue of the present invention (upper) and an exemplary result of M-mode analysis (lower). The left portion shows an, while the right portion shows the infarcted heart after treatment.

20

Figure 19 shows exemplary results of a test for the cardiac function of a prosthetic myoblast tissue of the present invention after implantation. The ejection fraction (EF), fractional shortening (FS), endo-systolic area (ESA), and E-Wave are shown. Diamonds indicate a prosthetic myoblast tissue, squares indicate myoblast injection, and triangles indicate a control.

25

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Figure 20 shows an example of comparison in wall



pressure between a prosthetic myoblast tissue of the present invention and myoblast. The results of photographs (upper left: prosthetic tissue; upper right: cell injection; lower right: control) are summarized to a lower left graph.

5

Figure 21 shows comparison between a prosthetic myoblast tissue of the present invention and a control visualized by desmin staining, Factor VIII staining, and GFP expression. The upper portion shows desmin staining (left: prosthetic tissue), GFP expression (right: prosthetic tissue), and GFP expression (middle: control). The lower portion shows Factor VIII staining (prosthetic tissue, cell injection, and control from the right).

10

Figures 22A to 22F show electrophysiological connection when a prosthetic tissue of the present invention is used. Figures 22A to 22C show a control, while Figures 22D to 22F show a prosthetic myoblast tissue. In the prosthetic myoblast tissue, electrophysiological connection is observed.

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Figures 23A to 23C show GFP expression when a prosthetic tissue of the present invention is used. GFP expression is shown by motion picture display. Figures 23A to 23C provide representative frames as still pictures.

25

Figures 24A to 24C show an exemplary ultrasound echography temporal analysis of treatment using a prosthetic tissue of the present invention. Results of ultrasound echography for an infarcted heart treated according to the present invention are shown by motion picture display. Figures 24A to 24C provide representative frames as still pictures.

30

Figures 25A to 25C show an exemplary ultrasound echography temporal analysis of treatment using a prosthetic tissue of the present invention. Figure 25A shows a control, while Figure 25B shows use of a prosthetic tissue of the present invention. Results of ultrasound echography are shown by motion picture display. Figures 25A to 25C provide representative frames as still pictures. The left portion shows an infarcted heart control, while the right portion shows a result of a myoblast sheet of the present invention.

Figure 26A shows an exemplary ultrasound echography analysis of treatment using a prosthetic tissue of the present invention. Figure 26A is a photograph showing the same sample as in Figures 25A to 25C at a different time point. Results of ultrasound echography are shown by motion picture display. Figure 26A provides representative frames as still pictures. The left portion shows an infarcted heart control, while the right portion shows a result of a myoblast sheet of the present invention.

Figure 26B shows an exemplary ultrasound echography analysis of treatment using a prosthetic tissue of the present invention. Figure 26B is a photograph showing the same sample as in Figures 25A to 25C at a different time point. Results of ultrasound echography are shown by motion picture display. Figure 26B provides representative frames as still pictures. The left portion shows an infarcted heart control, while the right portion shows a result of a myoblast sheet of the present invention.

Figure 26C shows an exemplary ultrasound echography analysis of treatment using a prosthetic tissue of the present

invention. Figure 26C is a photograph showing the same sample as in Figures 25A to 25C at a different time point. Results of ultrasound ecography are shown by motion picture display. Figure 26C provides representative frames as still pictures. The left portion shows an infarcted heart control, while the right portion shows a result of a myoblast sheet of the present invention.

Figures 27A to 27C show an exemplary ultrasound echography analysis of treatment using a prosthetic tissue of the present invention. Results of ultrasound ecography for an infarcted heart treated according to the present invention are shown by motion picture display. Figures 27A to 27C provide representative frames as still pictures.

Figure 28 shows cell affinity by RT-PCR.

Figure 29 shows a change in an implanted myoblast sheet after implantation.

Figure 30A shows an example of Masson's Trichrome staining of a prosthetic tissue of the present invention using myoblasts, which was applied to a cardiomyopathic hamster. The upper portion shows sheet (prosthetic tissue) implantation, cell implantation, and a control ( $\times 10$ ). The lower portion shows an enlarged view for each ( $\times 40$ ).

Figure 30B shows histology (myoblast sheet is accepted by a dilated cardiomyopathic heart and enlarges a ventricular wall).

Figure 30C shows comparison of expression levels of  $\alpha$ -sarcoglycan (myoblast sheet implantation enhances

expression of  $\alpha$ -sarcoglycan).

Figure 30D shows comparison of expression levels of  $\beta$ -sarcoglycan (myoblast sheet implantation enhances expression of  $\beta$ -sarcoglycan).

Figure 31 shows a survival rate of cardiomyopathic hamsters into which a prosthetic tissue (sheet) comprising myoblasts of the present invention has been implanted myoblast. Injection of cells as they are is compared with administration of a prosthetic tissue.

Figure 32 shows electrical characteristics after implantation of a prosthetic tissue of the present invention. The left portion shows a prosthetic tissue comprising cardiomyocytes, while the right portion shows a prosthetic tissue comprising myoblasts.

Figure 33A shows a therapy for dilated cardiomyopathic hamsters using a prosthetic tissue of the present invention. The left portion shows EF, while the right portion shows HE staining. The lower right portion shows Masson's Trichrome staining.

Figure 33B shows a result of echocardiography 48 weeks after implantation (myoblast sheet implantation ameliorates the cardiac function of a dilated cardiomyopathy heart).

Figure 33C shows a result of echocardiography (the contractility of a left ventricle) 48 weeks after implantation (myoblast sheet implantation ameliorates the contractility of the left ventricle with dilated

cardiomyopathy).

Figure 34 shows an example of a therapy for a pig  
infarct model using a prosthetic tissue of the present  
5 invention.

Figure 35 shows a therapeutic effect  
(contractility) in a pig infarct model using a prosthetic  
tissue of the present invention.  
10

Figure 36 shows a therapeutic effect  
(expansibility) in a pig infarct model using a prosthetic  
tissue of the present invention.

Figure 37 shows a sheet produced by a prosthetic  
tissue production method without ascorbic acid.  
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Figure 38 shows a sheet produced by a prosthetic  
tissue production method with ascorbic acid according to  
the present invention.  
20

Figure 39 shows a sheet produced by a prosthetic  
tissue production method with ascorbic acid according to  
the present invention (HE staining).  
25

Figure 40 shows a technique for measuring stress and  
distortion characteristics to determine tensile strength.

Figure 41 shows a method for obtaining a load/removal  
of a load curve.  
30

Figure 42 shows a state of a tissue obtained by  
culturing synovial cells in the presence of ascorbic acid

2-phosphate.

(Description of Sequencing List)

5       SEQ ID NO. 1 sets forth a nucleic acid sequence of  
myosin heavy chain IIa (human: Accession No. NM\_017534).

      SEQ ID NO. 2 sets forth an amino acid sequence of  
myosin heavy chain IIa (human: Accession No. NM\_017534).

10       SEQ ID NO. 3 sets forth a nucleic acid sequence of  
myosin heavy chain IIb (human: Accession No. NM\_017533).

      SEQ ID NO. 4 sets forth an amino acid sequence of  
myosin heavy chain IIb (human: Accession No. NM\_017533).

15       SEQ ID NO. 5 sets forth a nucleic acid sequence of  
myosin heavy chain IIId(IIx) (human: Accession  
No. NM\_005963).

20       SEQ ID NO. 6 sets forth an amino acid sequence of  
myosin heavy chain IIId(IIx) (human: Accession  
No. NM\_005963).

      SEQ ID NO. 7 sets forth a nucleic acid sequence of  
25   CD56 (human: Accession No. U63041).

      SEQ ID NO. 8 sets forth an amino acid sequence of  
CD56 (human: Accession No. U63041).

30       SEQ ID NO. 9 sets forth a nucleic acid sequence of  
human MyoD (GENBANK Accession No. X56677).

      SEQ ID NO. 10 sets forth a polypeptide sequence

encoded by the nucleic acid sequence set forth in SEQ ID NO. 2.

5           SEQ ID NO. 11 sets forth a nucleic acid sequence of human myogenic factor 5 (MYF5) (GENBANK Accession No. NM\_005593).

10           SEQ ID NO. 12 sets forth a polypeptide sequence encoded by the nucleic acid sequence set forth in SEQ ID NO. 3.

15           SEQ ID NO. 13 sets forth a nucleic acid sequence of human myogenin (myogenic factor 4) (GENBANK Accession No. BT007233).

            SEQ ID NO. 14 sets forth a polypeptide sequence encoded by the nucleic acid sequence set forth in SEQ ID NO. 5.

20           SEQ ID NO. 15 sets forth a forward primer in RT-PCR for SRY.

            SEQ ID NO. 16 sets forth a reverse primer in RT-PCR for SRY.

25           SEQ ID NO. 17 sets forth a probe in RT-PCR for SRY.

            SEQ ID NO. 18 sets forth a forward primer in RT-PCR for IL2.

30           SEQ ID NO. 19 sets forth a reverse primer in RT-PCR for IL3.

SEQ ID NO. 20 sets forth a probe in RT-PCR for IL2.

#### BEST MODE FOR CARRYING OUT THE INVENTION

5           The present invention will be described below. It should be understood throughout the present specification that articles for singular forms include the concept of their plurality unless otherwise mentioned. Therefore, articles or adjectives for singular forms (e.g., "a", "an", "the",  
10           and the like in English) include the concept of their plurality unless otherwise specified. Also, it should be also understood that terms as used herein have definitions ordinarily used in the art unless otherwise mentioned. Therefore, all technical and scientific terms used herein  
15           have the same meanings as commonly understood by those skilled in the relevant art. Otherwise, the present application (including definitions) takes precedence.

#### (Definition of terms)

20           The definitions of specific terms used herein are described below.

#### (Regenerative medicine)

25           As used herein, the term "regeneration" refers to a phenomenon in which when an individual organism loses a portion of tissue, the remaining tissue grows and recovers. The extent or manner of regeneration varies depending among animal species or among tissues in the same individual. Most human tissues have limited regeneration capability, and  
30           therefore, complete regeneration is not expected if a large portion of tissue is lost. In the case of severe damage, a tissue may grow which has strong proliferation capability different from that of lost tissue, resulting in incomplete



regeneration where the damaged tissue is incompletely regenerated and the function of the tissue cannot be recovered. In this case, a structure made of a bioabsorbable material is used to prevent a tissue having strong proliferation capability from infiltrating the defective portion of the tissue so as to secure space for proliferation of the damaged tissue. Further, by supplementing with a cell growth factor, the regeneration capability of the damaged tissue is enhanced. Such a regeneration technique is applied to cartilages, bones, and peripheral nerves, for example. It has been so far believed that nerve cells and cardiac muscles have no or poor regeneration capability. Recently, it was reported that there are tissue stem cells (somatic stem cells), which have both the capability of differentiating into these tissues and self-proliferation capability. Expectations are running high for regenerative medicine using tissue stem cells. Embryonic stem cells (ES cells) are cells, which have the capability of differentiating into all tissues. Efforts have been made to use ES cells for regeneration of complicated organs, such as kidney, liver, and the like, but have not yet been realized.

The term "cell" is herein used in its broadest sense in the art, referring to a structural unit of tissue of a multicellular organism, which is capable of self-replicating, has genetic information and a mechanism for expressing it, and is surrounded by a membrane structure which isolates the living body from the outside. In the method of the present invention, any cell can be used as a subject. The number of cells used in the present invention can be counted through an optical microscope. When counting using an optical microscope, the number of nuclei is counted. Tissues are sliced into tissue sections, which are then stained with

hematoxylin-eosin (HE) to variegate nuclei derived from extracellular matrices (e.g., elastin or collagen) and cells. These tissue sections are observed under an optical microscope and the number of nuclei in a particular area  
5 (e.g., 200  $\mu\text{m}$   $\times$  200  $\mu\text{m}$ ) can be estimated to be the number of cells. Cells used herein may be either naturally-occurring cells or artificially modified cells (e.g., fusion cells, genetically modified cells, etc.). Examples of cell sources include, but are not limited to,  
10 a single-cell culture; the embryo, blood, or body tissue of a normally-grown transgenic animal; a cell mixture of cells derived from normally-grown cell lines; and the like.

As used herein, the term "stem cell" refers to a cell  
15 capable of self replication and pluripotency. Typically, stem cells can regenerate an injured tissue. Stem cells used herein may be, but are not limited to, embryonic stem (ES) cells or tissue stem cells (also called tissular stem cell, tissue-specific stem cell, or somatic stem cell). A stem  
20 cell may be an artificially produced cell (e.g., fusion cells, reprogrammed cells, or the like used herein) as long as it can have the above-described abilities. Embryonic stem cells are pluripotent stem cells derived from early embryos. An embryonic stem cell was first established in 1981, and  
25 has been applied to production of knockout mice since 1989. In 1998, a human embryonic stem cell was established, which is currently becoming available for regenerative medicine. Tissue stem cells have a relatively limited level of differentiation unlike embryonic stem cells. Tissue stem  
30 cells are present in tissues and have an undifferentiated intracellular structure. Tissue stem cells have a higher nucleus/cytoplasm ratio and have few intracellular organelles. Most tissue stem cells have pluripotency, along

cell cycle, and proliferative ability beyond the life of the individual. As used herein, stem cells may be preferably embryonic stem cells, though tissue stem cells may also be employed depending on the circumstance.

5

Tissue stem cells are separated into categories of sites from which the cells are derived, such as the dermal system, the digestive system, the bone marrow system, the nervous system, and the like. Tissue stem cells in the dermal system include epidermal stem cells, hair follicle stem cells, and the like. Tissue stem cells in the digestive system include pancreatic (common) stem cells, hepatic stem cells, and the like. Tissue stem cells in the bone marrow system include hematopoietic stem cells, mesenchymal stem cells, and the like. Tissue stem cells in the nervous system include neural stem cells, retinal stem cells, and the like.

As used herein, the term "somatic cell" refers to any cell other than a germ cell, such as an egg, a sperm, or the like, which does not transfer its DNA to the next generation. Typically, somatic cells have limited or no pluripotency. Somatic cells used herein may be naturally-occurring or genetically modified as long as they can achieve the intended treatment.

25

The origin of a stem cell is categorized into the ectoderm, endoderm, or mesoderm. Stem cells of ectodermal origin are mostly present in the brain, including neural stem cells. Stem cells of endodermal origin are mostly present in bone marrow, including blood vessel stem cells, hematopoietic stem cells, mesenchymal stem cells, and the like. Stem cells of mesoderm origin are mostly present in organs, including hepatic stem cells, pancreatic stem cells,

30

and the like. As used herein, somatic cells may be derived from any mesenchyme. Preferably, somatic cells derived from mesenchyme may be employed.

5           As cells for use in construction of a prosthetic tissue or three-dimensional structure of the present invention, differentiated cells or stem cells derived from the above-described ectoderm, endoderm, or mesoderm may be employed, for example. Examples of such cells include  
10   mesenchymal cells. In a certain embodiment, as such cells, myoblasts (e.g., skeletal myoblast, etc.), fibroblasts, synovial cells, and the like may be employed. As such cells, differentiated cells or stem cells can be used as they are. Cells differentiated from stem cells into a desired direction  
15   can be used.

          As used herein, the term "mesenchymal stem cell" refers to a stem cell found in mesenchyme. The term "mesenchymal stem cell" may be herein abbreviated as "MSC".  
20   Mesenchyme refers to a population of free cells which are in the asterodal shape or have irregular projections and bridge gaps between epithelial tissues, and which are recognized in each stage of development of multicellular animals. Mesenchyme also refers to tissue formed with  
25   intracellular cement associated with the cells. Mesenchymal stem cells have proliferation ability and the ability to differentiate into bone cells, cartilage cells, muscle cells, stroma cells, tendon cells, and fat cells. Mesenchymal stem cells are employed in order to culture or  
30   grow bone marrow cells or the like collected from patients, or differentiate them into cartilage cells or osteoblasts. Mesenchymal stem cells are also employed as reconstruction material, such as alveolar bones; bones, cartilages or joints

for arthropathy or the like; and the like. There is a large demand for mesenchymal stem cells. Also, mesenchymal stem cells can be differentiated into blood cells and lymphoid cells. Therefore, there is an increasing demand for mesenchymal stem cells. A prosthetic tissue or three-dimensional structure of the present invention comprising mesenchymal stem cells or differentiated mesenchymal stem cells is particularly useful when a structure is required in these applications.

As used herein, the term "isolated" means that naturally accompanying material is at least reduced, or preferably substantially completely eliminated, in normal circumstances. Therefore, the term "isolated cell" refers to a cell substantially free from other accompanying substances (e.g., other cells, proteins, nucleic acids, etc.) in natural circumstances. The term "isolated tissue" refers to a tissue substantially free from substances other than that tissue (e.g., in the case of prosthetic tissues, substances, scaffolds, sheets, coats, etc. used when the prosthetic tissue is produced). The term "isolated" in relation to nucleic acids or polypeptides means that, for example, the nucleic acids or the polypeptides are substantially free from cellular substances or culture media when they are produced by recombinant DNA techniques; or precursory chemical substances or other chemical substances when they are chemically synthesized. Isolated nucleic acids are preferably free from sequences naturally flanking the nucleic acid within an organism from which the nucleic acid is derived (i.e., sequences positioned at the 5' terminus and the 3' terminus of the nucleic acid).

As used herein, the term "intact" in relation to

prosthetic tissues, three-dimensional structures, and the like, refers to no physical external injury. For example, when a prosthetic tissue or the like is produced and is then separated from a circumstance in which the production has  
5 been conducted, it has substantially no external injury, such as physical impact or the like.

As used herein, the term "established" in relation to cells refers to a state of a cell in which a particular  
10 property (pluripotency) of the cell is maintained and the cell undergoes stable proliferation under culture conditions. Therefore, established stem cells maintain pluripotency.

As used herein, the term "non-embryonic" refers to  
15 not being directly derived from early embryos. Therefore, the term "non-embryonic" refers to cells derived from parts of the body other than early embryos. Also, modified embryonic stem cells (e.g., genetically modified or fusion embryonic stem cells, etc.) are encompassed by non-embryonic  
20 cells.

As used herein, the term "differentiated cell" refers to a cell having a specialized function and form (e.g., muscle cells, neurons, etc.). Unlike stem cells, differentiated  
25 cells have no or little pluripotency. Examples of differentiated cells include epidermic cells, pancreatic parenchymal cells, pancreatic duct cells, hepatic cells, blood cells, cardiac muscle cells, skeletal muscle cells, osteoblasts, skeletal myoblasts, neurons, vascular  
30 endothelial cells, pigment cells, smooth muscle cells, fat cells, bone cells, cartilage cells, and the like.

As used herein, the term "tissue" refers to a group

of cells having the same function and form in cellular organisms. In multicellular organisms, constituent cells are usually differentiated so that the cells have specialized functions, resulting in division of labor. Therefore, 5 multicellular organisms are not simple cell aggregations, but constitute organic or social cell groups having a certain function and structure. Examples of tissues include, but are not limited to, integument tissue, connective tissue, muscular tissue, nervous tissue, and the like. Tissue 10 targeted by the present invention may be derived from any organ or part of an organism. In a preferred embodiment of the present invention, tissue targeted by the present invention includes, but is not limited to, blood vessels, blood vessel-like tissue, cardiac valves, pericardia, dura 15 mater, cornea, joints, and bones.

As used herein, the term "prosthetic tissue" refers to tissue having a state different from natural states. Typically, a prosthetic tissue is herein prepared by cell 20 culture. Tissue which is removed from an organism and is not subjected to any treatment is not referred to as a prosthetic tissue. A prosthetic tissue may include materials derived from organisms and materials not derived from organisms. The prosthetic tissue of the present 25 invention typically comprises a cell and/or a biological material, and may comprise other materials. More preferably, a prosthetic tissue of the present invention is composed substantially only of a cell and/or a biological material. Such a biological material is preferably derived from cells 30 constituting the tissue (e.g., extracellular matrix, etc.).

As used herein, the term "implantable prosthetic tissue" refers to a prosthetic tissue, which can be used

for actual clinical implantation and can function as a tissue at the implantation site for a certain period of time after implantation. Implantable prosthetic tissue typically has sufficient strength, sufficient size, sufficient nonporousness, sufficient thickness, sufficient biocompatibility, sufficient affinity, and the like.

The sufficient strength of an implantable prosthetic tissue varies depending on a part targeted by implantation, but can be determined as appropriate by those skilled in the art. However, an implantable prosthetic tissue preferably has at least a certain level of strength. Such a level of strength (e.g., tensile strength) is at least about 50% of the natural strength of a part targeted by implantation, preferably at least about 60%, more preferably about 70%, even more preferably about 80%, and most preferably at least about 100%. The strength can be measured by measuring stress or distortion characteristics or conducting a creep characteristics indentation test as described below.

20

The sufficient size of an implantable prosthetic tissue varies depending on a part targeted by implantation, but can be determined as appropriate by those skilled in the art. However, an implantable prosthetic tissue preferably has at least a certain size. Such a size (e.g., area) is at least 1 cm<sup>2</sup>, preferably at least 2 cm<sup>2</sup>, more preferably at least 3 cm<sup>2</sup>, even more preferably at least 4 cm<sup>2</sup>, at least 5 cm<sup>2</sup>, at least 6 cm<sup>2</sup>, at least 7 cm<sup>2</sup>, at least 8 cm<sup>2</sup>, at least 9 cm<sup>2</sup>, at least 10 cm<sup>2</sup>, at least 15 cm<sup>2</sup>, or at least 20 cm<sup>2</sup>.

30

The sufficient nonporousness of an implantable prosthetic tissue varies depending on a part targeted by



implantation, but can be determined as appropriate by those skilled in the art. As used herein, the term "nonporousness" refers to a state lacking pore(s). Here, the pore refers to a hole having a substantial size such that body fluid or its equivalent (e.g., an aqueous solution, etc.) leaks from a prosthetic tissue. Therefore, nonporousness can be determined as follows. A prosthetic tissue is placed horizontally. Body fluid or its equivalent is placed on the tissue. It is observed whether or not the body fluid or its equivalent leaks from the tissue. If there is no leak, the tissue is judged to have nonporousness.

The sufficient thickness of an implantable prosthetic tissue varies depending on a part targeted by implantation, but can be determined as appropriate by those skilled in the art. However, an implantable prosthetic tissue preferably has at least a certain thickness. Such a thickness is typically at least about 50  $\mu\text{m}$ , preferably at least about 100  $\mu\text{m}$ , more preferably about 150  $\mu\text{m}$ , even more preferably at least about 200  $\mu\text{m}$ , at least about 300  $\mu\text{m}$ , at least about 400  $\mu\text{m}$ , at least about 500  $\mu\text{m}$ , at least about 600  $\mu\text{m}$ , at least about 700  $\mu\text{m}$ , at least about 800  $\mu\text{m}$ , at least about 900  $\mu\text{m}$ , at least about 1 mm. When an implantable prosthetic tissue is implanted into the heart, the tissue may only have these minimum thicknesses. When implantable prosthetic tissue is used in other applications, the tissue may preferably have a greater thickness. In such a case, for example, implantable prosthetic tissue has preferably a thickness of at least 2 mm, more preferably at least 3 mm, and even more preferably 5 mm.

The sufficient biocompatibility of implantable prosthetic tissue varies depending on a part targeted by

implantation, but can be determined as appropriate by those skilled in the art. However, an implantable prosthetic tissue preferably has at least a certain level of biocompatibility. Typically, a desired level of biocompatibility is, for example, such that biological connection to surrounding tissues is achieved without any inflammation, any immune reaction or the like. The present invention is not limited to this. In some cases (e.g., corneas, etc.), an immune reaction is less likely to occur.

Therefore, an implantable prosthetic tissue has biocompatibility to an extent, which achieves the object of the present invention even when an immune reaction is likely to occur in other organs. Examples of parameters indicating biocompatibility include, but are not limited to, the presence or absence of an extracellular matrix, the presence or absence of an immune reaction, the degree of inflammation, and the like. Such biocompatibility can be determined by examining the compatibility of a prosthetic tissue at an implantation site after implantation (e.g., confirming that an implanted prosthetic tissue is not destroyed). See "Hito Ishoku Zoki Kyozeitsu Hanno no Byori Soshiki Shindan Kijyun Kanbetsu Shindan to Seiken Hyohon no Toriatsukai (Zufu) Jinzo Ishoku, Kanzo Ishoku Oyobi Shinzo Ishoku [Pathological Tissue Diagnosis Criterion for Human Transplanted Organ Rejection Reaction Handling of Differential Diagnosis and Biopsy Specimen (Illustrated Book) Kidney Transplantation, Liver Transplantation and Heart Transplantation]" The Japan Society for Transplantation and The Japanese Society for Pathology editors, Kanehara Shuppan Kabushiki Kaisha (1998). According to this document, biocompatibility is divided into Grade 0, 1A, 1B, 2, 3A, 3B, and 4. At Grade 0 (no acute rejection), no acute rejection reaction, cardiomyocyte

failure, or the like is found in biopsy specimens. At Grade 1A (focal, mild acute rejection), there is focal infiltration of large lymphocytes around blood vessels or into interstitial tissue, while there is no damage to cardiomyocytes. This observation is obtained in one or a plurality of biopsy specimens. At Grade 1B (diffuse, mild acute rejection), there is diffuse infiltration of large lymphocytes around blood vessels or into interstitial tissue or both, while there is no damage to cardiomyocytes. At Grade 2 (focal, moderate acute rejection), there is a single observed infiltration focus of inflammatory cells clearly bordered from the surrounding portions. Inflammation cells are large activated lymphocytes and may include eosinophils. Damage to cardiomyocytes associated with modification of cardiac muscle is observed in lesions. At Grade 3A (multifocal, moderate acute rejection), there are multiple infiltration foci of inflammatory cells which are large activated lymphocytes and may include eosinophils. Two or more of the multiple inflammatory infiltration foci of inflammatory cells have damages to cardiomyocytes. In some cases, there is also rough infiltration of inflammatory cells into the endocardium. The infiltration foci are observed in one or a plurality of biopsy specimens. At Grade 3B (multifocal, borderline severe acute rejection), there are more confluent and diffuse infiltration foci of inflammatory cells found in more biopsy specimens than those observed at Grade 3A. There is infiltration of inflammatory cells including large lymphocytes and eosinophils, in some cases neutrophils, as well as damage to cardiomyocytes. There is no hemorrhage. At Grade 4 (severe acute rejection), there is infiltration of various inflammatory cells including activated lymphocytes, eosinophils, and neutrophils. There is always damage to cardiomyocytes and necrosis of

cardiomyocytes. Edema, hemorrhage, and/or angitis are also typically observed. Infiltration of inflammatory cells into the endocardium, which is different from the "Quilty" effect, is typically observed. When a therapy is strongly  
5 conducted using an immunosuppressant for a considerably long period of time, edema and hemorrhage may be more significant than infiltration.

The sufficient affinity of an implantable prosthetic  
10 tissue varies depending on a part targeted by implantation, but can be determined as appropriate by those skilled in the art. Examples of parameters for affinity include, but are not limited to, biological connection ability between an implanted prosthetic tissue and its implantation site,  
15 and the like. Such affinity can be determined based on the presence of biological connection at an implantation site after implantation. Preferable affinity is herein such that an implanted prosthetic tissue has the same function as that of a site in which the tissue is implanted, for example.

20

As used herein, the term "membranous tissue" refers to a tissue in the form of membrane and is also referred to as "planar tissue". Examples of membranous tissue include a portion of tissue having a certain area of an organ (e.g.,  
25 pericardium, dura mater, cornea, etc.) or bag-shaped tissue, and the like.

As used herein, the term "organ" refers to a structure which is a specific part of an individual organism where  
30 a certain function of the individual organism is locally performed and which is morphologically independent. Generally, in multicellular organisms (e.g., animals and plants), organs are made of several tissues in specific

spatial arrangement and tissue is made of a number of cells. Examples of such organs include, but are not limited to, skin, blood vessel, cornea, kidney, heart, liver, umbilical cord, intestine, nerve, lung, placenta, pancreas, brain, joint, bone, cartilage, peripheral limbs, retina, and the like. Examples of such organs include, but are not limited to, organs of the skin system, the parenchyma pancreas system, the pancreatic duct system, the hepatic system, the blood system, the myocardial system, the skeletal muscle system, the osteoblast system, the skeletal myoblast system, the nervous system, the blood vessel endothelial system, the pigment system, the smooth muscle system, the fat system, the bone system, the cartilage system, and the like.

As used herein, the term "bag-shaped organ" refers to an organ which has a three-dimensional expanse and the inside of which may be connected via a tubular tissue to the outside. Examples of bag-shaped organs include, but are not limited to, heart, liver, kidney, stomach, spleen, and the like.

In one embodiment, the present invention targets vascular system-related organs, and preferably ischemic organs (e.g., heart having myocardial infarcted heart having ischemia, etc.). In a preferred embodiment, the present invention targets blood vessels, blood vessel-like tissue, heart, heart valves, pericardia, dura mater, cornea, and bones. In another preferred embodiment, the present invention targets heart, heart valves, pericardia, and blood vessels.

As used herein, the term "wrap" in relation to a prosthetic tissue, a three-dimensional structure, or the

like, which is wrapped around a certain part (e.g., an injured site, etc.), means that the prosthetic tissue or the like is arranged so as to cover the part (i.e., conceal an injury or the like). The terms "wrap" and "arrange (or locate) so as to cover" are used interchangeably. By observing the spatial relationship between the part and the prosthetic tissue or the like, it can be determined whether or not the part is covered by the prosthetic tissue or the like. In a preferred embodiment, in a wrapping step, a prosthetic tissue or the like can be wrapped one turn around a certain site.

A "sufficient time required for a prosthetic tissue to biologically join with a part" herein varies depending on a combination of the part and the prosthetic tissue, but can be determined as appropriate by those skilled in the art based on the combination. Examples of such a time include, but are not limited to, 1 week, 2 weeks, 1 month, 2 months, 3 months, 6 months, 1 year, and the like, after operation. In the present invention, a prosthetic tissue preferably comprises substantially only cells and materials derived from the cells, and therefore, there is no particular material which needs to be extracted after operation. Therefore, the lower limit of the sufficient time is not particularly important. Thus, in this case, a longer time is more preferable. If the time is substantially extremely long, reinforcement is substantially completed.

As used herein, the term "immune reaction" refers to a reaction due to the dysfunction of immunological tolerance between a graft and a host. Examples of immune reactions include, but are not limited to, a hyperacute rejection reaction (within several minutes after

implantation) (immune reaction caused by antibodies, such as  $\beta$ -Gal or the like), an acute rejection reaction (reaction caused by cellular immunity about 7 to 21 days after implantation), a chronic rejection reaction (rejection reaction caused by cellular immunity 3 or more months after operation), and the like.

As used herein, the elicitation of an immune reaction can be confirmed by pathological and histological examination of the type, number, or the like of infiltration of (immunological) cells into implanted tissue using staining (e.g., HE staining, etc.), immunological staining, or microscopic inspection of tissue sections.

As used herein, the term "calcification" refers to precipitation of calcareous substances in organisms.

As used herein, "calcification" *in vivo* can be determined by measuring calcium concentration. Specifically, implanted tissue is taken out; the tissue section is dissolved by acid treatment or the like; and the atomic absorption of the solution is measured by a trace element quantifying device.

As used herein, the term "within organism(s) (or in organism(s))" or "*in vivo*" refers to the inner part of organism(s). In a specific context, "within organism(s)" refers to a position at which a subject tissue or organ is placed.

As used herein, "*in vitro*" indicates that a part of an organism is extracted or released outside the organism for various purposes of research (e.g., in a test tube).

The term *in vitro* is in contrast to the term *in vivo*.

As used herein, the term "*ex vivo*" refers to a series of operations where target cells into which a gene will be introduced are extracted from a subject; a therapeutic gene is introduced *in vitro* into the cells; and the cells are returned into the same subject.

As used herein, the term "material derived from cell(s)" refers to any material originating from the cell(s), including, but not being limited to, materials constituting the cell(s), materials secreted by the cell(s), materials metabolized by the cell(s), and the like. Representative examples of materials derived from cells include, but are not limited to, extracellular matrices, hormones, cytokines, and the like. Materials derived from cells typically have substantially no adverse effect on the cells and their hosts. Therefore, when the material is contained in a prosthetic tissue, a three-dimensional structure, or the like, the material typically has substantially no adverse effect on the prosthetic tissue, three-dimensional structure, or the like.

As used herein, the term "extracellular matrix" (ECM) refers to a substance existing between somatic cells no matter whether the cells are epithelial cells or non-epithelial cells. Extracellular matrices are typically produced by cells, and therefore, are biological materials. Extracellular matrices are involved in supporting tissue as well as in internal environmental structure essential for survival of all somatic cells. Extracellular matrices are generally produced from connective tissue cells. Some extracellular matrices are secreted from cells possessing



basal membrane, such as epithelial cells or endothelial cells. Extracellular matrices are roughly divided into fibrous components and matrices filling there between. Fibrous components include collagen fibers and elastic fibers. A  
5 basic component of matrices is a glycosaminoglycan (acidic mucopolysaccharide), most of which is bound to non-collagenous protein to form a polymer of a proteoglycan (acidic mucopolysaccharide-protein complex). In addition, matrices include glycoproteins, such as laminin of basal  
10 membrane, microfibrils around elastic fibers, fibers, fibronectins on cell surfaces, and the like. Particularly differentiated tissue has the same basic structure. For example, in hyaline cartilage, chondroblasts characteristically produce a large amount of cartilage  
15 matrices including proteoglycans. In bones, osteoblasts produce bone matrices which cause calcification. In one embodiment of the present invention, the prosthetic tissue, three-dimensional structure, or the like of the present invention may be advantageously similar to the composition  
20 of an extracellular matrix (e.g., elastin, collagen (e.g., Type I, Type IV, etc.), laminin, etc.) of a site of an organ for which implantation is intended. In the present invention, extracellular matrices include cell adhesion molecules. As used herein, the terms "cell adhesion molecule" and "adhesion  
25 molecule" are used interchangeably, referring to a molecule capable of mediating the joining of two or more cells (cell adhesion) or adhesion between a substrate and a cell. In general, cell adhesion molecules are divided into two groups: molecules involved in cell-cell adhesion (intercellular  
30 adhesion) (cell-cell adhesion molecules) and molecules involved in cell-extracellular matrix adhesion (cell-substrate adhesion) (cell-substrate adhesion molecules). A prosthetic tissue or three-dimensional

structure of the present invention typically comprises such a cell adhesion molecule. Therefore, cell adhesion molecules herein include a protein of a substrate and a protein of a cell (e.g., integrin, etc.) in cell-substrate adhesion.

5 A molecule other than proteins falls within the concept of cell adhesion molecule as long as it can mediate cell adhesion.

For cell-cell adhesion, cadherin, a number of molecules belonging in an immunoglobulin superfamily (NCAM1, 10 ICAM, fasciclin II, III, etc.), selectin, and the like are known, each of which is known to join cell membranes via a specific molecular reaction. Therefore, in one embodiment, the prosthetic tissue, three-dimensional structure, or the like of the present invention preferably has substantially 15 the same composition of cadherin, immunoglobulin superfamily molecules, or the like as that of a site for which implantation is intended.

Thus, various molecules are involved in cell adhesion 20 and have different functions. Those skilled in the art can appropriately select a molecule to be contained in a prosthetic tissue or three-dimensional structure of the present invention depending on the purpose. Techniques for cell adhesion are well known as described above and as 25 described in, for example, "Saibogaimatorikkusu-Rinsho heno Oyo- [Extracellular matrix -Clinical Applications-], Medical Review.

It can be determined whether or not a certain molecule 30 is a cell adhesion molecule, by an assay, such as biochemical quantification (an SDS-PAG method, a labeled-collagen method, etc.), immunological quantification (an enzyme antibody method, a fluorescent antibody method, an immunohistological

study, etc.), a PCR method, a hybridization method, or the like, in which a positive reaction is detected. Examples of such a cell adhesion molecule include, but are not limited to, collagen, integrin, fibronectin, laminin, vitronectin, 5 fibrinogen, an immunoglobulin superfamily member (e.g., CD2, CD4, CD8, ICM1, ICAM2, VCAM1), selectin, cadherin, and the like. Most of these cell adhesion molecules transmit into a cell an auxiliary signal for cell activation due to intercellular interaction as well as cell adhesion. 10 Therefore, an adhesion factor for use in an implant of the present invention preferably transmits an auxiliary signal for cell activation into a cell. This is because cell activation can promote growth of cells originally present or aggregating in a tissue or organ at an injured site after application of an implant thereto. It can be determined 15 whether or not such an auxiliary signal can be transmitted into a cell, by an assay, such as biochemical quantification (an SDS-PAG method, a labeled-collagen method, etc.), immunological quantification (an enzyme antibody method, a fluorescent antibody method, an immunohistological study, 20 etc.), a PDR method, a hybridization method, or the like, in which a positive reaction is detected.

An example of a cell adhesion molecule is cadherin 25 which is present in many cells capable of being fixed to tissue. Cadherin can be used in a preferred embodiment of the present invention. Examples of a cell adhesion molecule in cells of blood and the immune system which are not fixed to tissue, include, but are not limited to, immunoglobulin 30 superfamily molecules (CD 2, LFA-3, ICAM-1, CD2, CD4, CD8, ICM1, ICAM2, VCAM1, etc.); integrin family molecules (LFA-1, Mac-1, gpIIbIIIa, p150, p95, VLA1, VLA2, VLA3, VLA4, VLA5, VLA6, etc.); selectin family molecules (L-selectin,

E-selectin, P-selectin, etc.), and the like. Therefore, such a molecule may be useful for treatment of a tissue or organ of blood and the immune system.

5           Nonfixed cells need to be adhered to a specific tissue in order to act on the tissue. In this case, it is believed that cell-cell adhesion is gradually enhanced via a first adhesion by a selectin molecule or the like which is constantly expressed and a second adhesion by a subsequently activated  
10           integrin molecule. Therefore, in the present invention, a cell adhesion molecule for mediating the first adhesion and another cell adhesion molecule for mediating the second adhesion may be used together.

15           As used herein, the term "tissue injury rate" refers to a parameter which indicates a function of a tissue or organ; an indicator which indicates how much a treated tissue or organ is injured; and an indicator which indicates whether or not a tissue or organ can have its original function.  
20           Methods for determining a tissue injury rate are known in the art. For example, a tissue injury rate can be determined by counting elastin ruptured sites. Herein, a visual field is divided into units of  $100\ \mu\text{m} \times 100\ \mu\text{m}$ . In each unit, the presence or absence of an elastin ruptured site is determined.  
25           If there is an elastin ruptured site in a unit, the count is incremented. One visual field has 24 units. The extracellular matrices of tissue sections stained by HE staining are subjected to microscopic inspection and counting. Non-treated tissue is defined as having a tissue injury rate  
30           of 0%. A tissue injury rate is calculated by  $x/24$ . In this case, non-treated tissue corresponds to  $x=0$ .

As used herein, the term "tissue strength" refers

to a parameter which indicates a function of a tissue or organ and a physical strength of the tissue or organ. Tissue strength can be generally determined by measuring tensile strength (e.g., break strength, modulus of rigidity, Young's modulus, etc.). Such a general tensile test is well known. By analyzing data obtained by a general tensile test, various data, such as break strength, modulus of rigidity, Young's modulus, and the like, can be obtained. These values can be herein used as indicators of tissue strength. Typically, tissue strength which allows clinical applications is herein required.

The tensile strength of a prosthetic tissue, three-dimensional structure, or the like of the present invention can be determined by measuring the stress and distortion characteristics thereof. Briefly, a load is applied to a sample; the resultant distortion and the load are input to respective A/D converters (e.g., ELK-5000) (1 ch: distortion, 2 ch: load); the stress and distortion characteristics are measured to determine the tensile strength of the sample (Figure 40). Tensile strength can also be determined by testing creep characteristics. A creep characteristics indentation test is conducted to investigate how a sample is extended over time while a constant load is applied to the sample. For small materials, thin materials, and the like, an indentation test is conducted using, for example, a triangular pyramid-shaped indenter with a tip having a radius of about 0.1  $\mu\text{m}$  to about 1  $\mu\text{m}$ . Initially, the indenter is pushed into a test piece so that a load is given to the test piece. When the indenter reaches from several tens of nanometers to several micrometers deep in the test piece, the indenter is drawn off to remove the load. Figure 41 shows a load/removal of load curve obtained by

the above-described test method. Rigidity, Young's modulus, or the like can be obtained based on the behavior of the load and the push depth derived from the curve.

5           In a preferred embodiment, the tensile strength of the prosthetic tissue or three-dimensional structure of the present invention is typically at least about 50% of the natural strength of a part in which implantation is intended, preferably at least about 60%, more preferably about 70%,  
10   even more preferably about 80%, and most preferably at least about 100%.

          In an alternative embodiment, the prosthetic tissue of the present invention may have a tissue strength of at  
15   least about 75% of that of a portion of natural tissue (e.g., a portion in which a clinical application is intended (e.g., heart, etc.)), preferably at least about 80%, more preferably at least about 85%, and even more preferably about 90%. The tissue strength of the prosthetic tissue may be equal to  
20   or greater than that of the natural tissue. The tissue strength of a natural tissue refers to a tissue strength which is possessed by a tissue of interest in its natural state. In addition to membranous tissue, other tissues (e.g., tubular tissue, etc.) preferably have a sufficiently strong  
25   tissue strength. In the case of tubular tissue, the tissue strength can be represented by a  $\beta$  value. A method for calculating a  $\beta$  value will be described in detail in another portion of the present specification and will also be illustrated in the Examples below. In a certain embodiment,  
30   the prosthetic tissue of the present invention has a tissue strength corresponding to a  $\beta$  value of at least about 15, preferably at least about 18, more preferably at least about 20, and even more preferably at least about 22. In another

embodiment, the prosthetic tissue of the present invention has a  $\beta$  value of at least about 75% of that of the tissue before treatment, preferably at least about 80%, more preferably at least about 85%, and even more preferably at least about 90%. The  $\beta$  value of the prosthetic tissue may be equal to or greater than that originally possessed by the untreated tissue. A characteristic (e.g., a  $\beta$  value) of untreated tissue refers to a characteristic of the tissue before treatment (e.g., treatment using 1,2-epoxide polymer in the present invention) (e.g., in the natural state). Therefore, for example, if an original tissue has a  $\beta$  value of 25, a prosthetic tissue of the present invention may preferably have a  $\beta$  value of at least 17.5, preferably at least 20, more preferably at least 21.25, and even more preferably at least 22.5.

As used herein, in the case of tubular tissue, the tissue strength can be represented by a rigidity parameter ( $\beta$  value). The  $\beta$  value can be calculated based on the following expression after the P-D (pressure-diameter) relationship is established:

$$\ln(P/P_s) = \beta(D/D_s - 1) \quad (1)$$

where  $P_s$  and  $D_s$  represents their standard values at 100 mmHg. The value of the diameter ( $D$ ) is measured under each  $P$  (pressure).

Both ends of a tubular tissue (e.g., a blood vessel, etc.) are each fixed to a pipe-like unit. The inside and outside of the tissue are filled with physiological saline. In this situation, pressure is applied to the inside of the tissue by an external device, while the outer diameter of

the tissue under the pressure is monitored. The measured pressure and outer diameter are substituted into expression (1) to calculate a  $\beta$  value (Sonoda H., Takamizawa K. et al., J. Biomed. Matr. Res., 2001: 266-276).

5

As used herein, the term "physiologically active substance" refers to a substance capable of acting on a cell or tissue. Physiologically active substances include cytokines and growth factors. A cellular physiologically active substance may be naturally-occurring or synthesized. Preferably, a cellular physiologically active substance is one that is produced by a cell or one that has a function similar thereto. As used herein, a cellular physiologically active substance may be in the form of a protein or a nucleic acid or in other forms. In actual practice, cellular physiologically active substances are typically proteins. In the present invention, a physiologically active substance may be used to promote the affinity of an implanted prosthetic tissue of the present invention, for example.

20

The term "cytokine" is used herein in the broadest sense in the art and refers to a physiologically active substance which is produced from a cell and acts on the same or different cell. Cytokines are generally proteins or polypeptides having a function of controlling an immune response, regulating the endocrine system, regulating the nervous system, acting against a tumor, acting against a virus, regulating cell growth, regulating cell differentiation, or the like. Cytokines are herein in the form of a protein or a nucleic acid or in other forms. In actual practice, cytokines are typically proteins.

30

The terms "growth factor" or "cell growth factor"



are used herein interchangeably and each refers to a substance which promotes or controls cell growth. Growth factors are also called "proliferation factors" or "development factors". Growth factors may be added to cell or tissue culture medium, substituting for serum macromolecules. It has been revealed that a number of growth factors have a function of controlling differentiation in addition to a function of promoting cell growth.

Examples of cytokines representatively include, but are not limited to, interleukins, chemokines, hematopoietic factors such as colony stimulating factors, a tumor necrosis factor, interferons, a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a fibroblast growth factor (FGF), a hepatocyte growth factor (HGF), an endothelial cell growth factor (VEGF), cardiotrophin, and the like, which have proliferative activity.

Cellular physiologically active substances, such as cytokines, growth factors, and the like, typically have redundancy in function. Accordingly, reference herein to a particular cytokine or growth factor by one name or function also includes any other names or functions by which the factor is known to those of skill in the art, as long as the factor has the activity of a cellular physiologically active substance for use in the present invention. Cytokines or growth factors can be used in a therapeutic or pharmaceutical agent according to a preferred embodiment of the present invention as long as they have preferable activity as described herein.

Therefore, in one embodiment of the present invention, it was revealed that when such a cytokine or growth factor

(e.g., HGF) is provided to an implantation site (e.g., an implantation site of the myocardium, etc.) concomitantly with a prosthetic tissue or three-dimensional structure of the present invention, the affinity of the prosthetic tissue or three-dimensional structure and an improvement in the function of the implantation site are observed. Thus, the present invention also provides such a combined therapy.

As used herein, the term "differentiation" refers to a developmental process of the state of the composite parts of organisms, such as cells, tissues, or organs and a process in which a characteristic tissue or organ is formed. The term "differentiation" is mainly used in embryology, developmental biology, and the like. In organisms, various tissues and organs are formed from divisions of a fertilized ovum (a single cell) to an adult. At early developmental stages (i.e., before cell division or after insufficient cell division), each cell or cell group has no morphological or functional feature and is not much distinguishable. Such a state is referred to as "undifferentiated". "Differentiation" may occur at the level of organs. A cell constituting an organ may develop into various cells or cell groups having different features. This phenomenon is also referred to as differentiation within an organ in the formation of the organ. Therefore, a prosthetic tissue or three-dimensional structure of the present invention may comprise a tissue including differentiated cells.

As used herein, the terms "implant", "graft", and "tissue graft" are used interchangeably, referring to homologous or heterologous tissue or a cell group, or an artificial material, which is inserted into a particular site of a body and thereafter forms a part of the body.

Therefore, a prosthetic tissue or three-dimensional structure of the present invention can be used as an implant. Examples of conventional grafts include, but are not limited to, organs or portions of organs, blood vessels, blood vessel-like tissue, heart, cardiac valves, pericardia, and the like. Therefore, grafts encompass any one of these which is inserted into a deficient part so as to compensate for the deficiency. Grafts include, but are not limited to, autografts, allografts, and xenografts, which depend on the type of their donor.

As used herein, the term "autograft" (a tissue, a cell, an organ, etc.) refers to a graft (a tissue, a cell, an organ, etc.) which is implanted into the same individual from which the graft is derived. As used herein, the term "autograft" (a tissue, a cell, an organ, etc.) may encompass a graft from a genetically identical individual (e.g. an identical twin) in a broad sense. As used herein, the terms "autologous" and "derived from a subject" are used interchangeably. Therefore, the term "not derived from a subject" in relation to a graft indicates that the graft is not autologous (i.e., heterologous).

As used herein, the term "allograft" (a tissue, a cell, an organ, etc.) refers to a graft (a tissue, a cell, an organ, etc.) which is implanted into an individual which is the same species but is genetically different from that from which the graft is derived. Since an allograft (a tissue, a cell, an organ, etc.) is genetically different from an individual (recipient) to which the graft is implanted, the graft may elicit an immune reaction. Such a graft (a tissue, a cell, an organ, etc.) includes, but is not limited to, for example, a graft (a tissue, a cell, an organ, etc.) derived

from a parent.

As used herein, the term "xenograft" (a tissue, a cell, an organ, etc.) refers to a graft (a tissue, a cell, an organ, etc.) which is implanted from a different species. Therefore, for example, when a human is a recipient, a porcine-derived graft (a tissue, a cell, an organ, etc.) is called a xenograft (a tissue, a cell, an organ, etc.).

As used herein, "recipient" (acceptor) refers to an individual which receives a graft (a tissue, a cell, an organ, etc.) or implanted matter (a tissue, a cell, an organ, etc.) and is also called "host". In contrast, an individual providing a graft (a tissue, a cell, an organ, etc.) or implanted matter (a tissue, a cell, an organ, etc.) is called "donor" (provider).

With a prosthetic tissue forming technique of the present invention, a prosthetic tissue derived from any cell can be used. This is because a prosthetic tissue (e.g., membranous tissues, organs, etc.) formed by the method of the present invention can exhibit a desired function while the tissue injury rate is maintained at a level which does not interfere with the therapy (i.e., a low level). Conventionally, tissues or organs are used as grafts without modification. In contrast to this, the present invention provides a tissue comprising three-dimensionally connected cells. Such a prosthetic three-dimensional tissue cannot be achieved by conventional techniques, and therefore, constitutes one significant effect of the present invention.

As used herein, the term "subject" refers to an organism to which treatment of the present invention is

applied and is also referred to as "patient". A patient or subject may be preferably a human.

Cells optionally used in a prosthetic tissue, three-dimensional structure, or tissue graft of the present invention may be derived from a syngeneic origin (self origin), an allogenic origin (non-self origin), or a heterologous origin. In view of rejection reactions, syngeneic cells are preferable. If rejection reactions do not raise problems, allogenic cells may be employed. Cells which elicit rejection reactions can be employed by optionally treating the cells in a manner that overcomes rejection reactions. Procedures for avoiding rejection reactions are known in the art (see, for example, "Shin Gekagaku Taikai, Dai 12 Kan, Zoki Ishoku (Shinzo Ishoku : Hai Ishoku Gijutsuteki, Rinriteki Seibi kara Jisshi ni Mukete [New Whole Surgery, Vol. 12, Organ Transplantation (Heart Transplantation · Lung Transplantation From Technical and Ethical Improvements to Practice)" (Revised 3rd ed.), Nakayama Shoten]. Examples of such methods include, but are not limited to, a method using immunosuppressants or steroidal drugs, and the like. For example, there are currently the following immunosuppressants for preventing rejection reactions: "cyclosporine" (SANDIMMUNE/NEORAL); "tacrolimus" (PROGRAF); "azathioprine" (IMURAN); "steroid hormone" (prednine, methylprednine); and "T-cell antibodies" (OKT3, ATG, etc.). A method which is used worldwide as a preventive immunosuppression therapy in many facilities, is the concurrent use of three drugs: cyclosporine, azathioprine, and steroid hormone. An immunosuppressant is desirably administered concurrently with a pharmaceutical agent of the present invention. The present invention is not limited to this. An immunosuppressant may be administered before

or after a regeneration/therapeutic method of the present invention as long as an immunosuppression effect can be achieved.

5           Cells used in the present invention may be derived from any organism (e.g., vertebrates and invertebrates). Preferably, cells derived from vertebrates are used. More preferably, cells derived from mammals (e.g., primates, rodents, etc.) are used. Even more preferably, cells derived  
10       from primates are used. Most preferably, cells derived from a human are used. Typically, cells from the same species as the host are preferably used.

          Examples of a subject treated by a prosthetic tissue  
15       of the present invention include, but are not limited to, the heart suffering from a heart disease (e.g., heart failure, ischemic heart diseases, myocardial infarct, cardiomyopathy, myocarditis, hypertrophic cardiomyopathy, dilated hypertrophic cardiomyopathy, and dilated cardiomyopathy);  
20       blood vessels in a pericardium patch, infarcted myocardium lower and upper limbs; and the like.

          Tissues targeted by the present invention may be any organ of an organism and may be derived from any organism.  
25       Examples of organisms targeted by the present invention include vertebrates and invertebrates. Preferably, organisms targeted by the present invention are mammals (e.g., primates, rodents, etc.). More preferably, organisms targeted by the present invention are primates. Most  
30       preferably, organisms targeted by the present invention are humans.

          When a prosthetic tissue, three-dimensional

structure, or the like of the present invention is used as a valve, techniques well known in the art for general use of prosthetic valves can be used to implant the prosthetic tissue or the like. For example, stentless heterologous tissue valves are well known in the art. For example, stentless heterologous tissue valves are known. In heterologous tissue valves, the presence of a stent reduces the effective area of the valve port, leading to calcification or degeneration of valve leaflets. Recently, by utilizing the morphology of the base portion of the porcine aorta, a stentless heterologous tissue valve stent without a stent has attracted attention as a prosthetic valve for the aortic valve (Gross C., et al., Ann. Thorac. Surg., 68:919, 1999). It is considered that the absence of a stent results in a small pressure difference across the valve even when a small-size valve is unavoidably used and is also effective for postoperative enlargement of the left ventricle. Further, the elasticity of the base portion of the aorta is maintained, stress to the cusp is small, and the durability can be expected to be improved as compared to a tissue valve with a stent. Further, stentless heterologous tissue valves can be used in the case of endocarditis due to infection or prosthetic valve infection. At present, substantially satisfactory intermediate-term postoperative results of stentless heterologous tissue valves have been reported in the USA and Europe, and long-term results can be expected to be satisfactory (Gross C., et al., Ann. Thorac. Surg., 68:919, 1999).

As used herein, the term "large size" in relation to a prosthetic tissue refers to the size of a portion thereof which has no pore. Representatively, the term "large size" means that the length in a longitudinal direction of a portion

having no pore is at least 1 cm, preferably at least 1.5 cm, and even more preferably at least 2 cm. In this case, the length in a transverse direction thereof is also at least 1 cm, preferably at least 1.5 cm, and even more preferably at least 2 cm. The present invention is not limited to this. When a "large size" is represented by the area of a prosthetic tissue, the area of an inscribed circle in a portion having no pore is typically at least 1 cm<sup>2</sup>, preferably at least 2 cm<sup>2</sup>, more preferably at least 3 cm<sup>2</sup>, even more preferably at least 4 cm<sup>2</sup>, still even more preferably at least 5 cm<sup>2</sup>, and most preferably at least 6 cm<sup>2</sup>.

As used herein, the term "flexibility" in relation to a prosthetic tissue refers to an ability to resist physical stimuli from external environments (e.g., pressure). A prosthetic tissue having flexibility is preferable when the implantation site moves or deforms autonomously or by external effects. Therefore, such a prosthetic tissue having flexibility preferably retains flexibility after implantation.

As used herein, the term "extendibility and contractibility" in relation to a prosthetic tissue refers to an ability to resist extending or contracting stimuli from external environments (e.g., pulsation). A prosthetic tissue having extendibility and contractibility is preferable when the implantation site is subjected to extending or contracting stimuli. Examples of implantation sites, which are subjected to extending or contracting stimuli, include, but are not limited to, heart, muscle, joint, cartilage, tendon, and the like. In one embodiment, extendibility and contractibility capable of withstanding the pulsation motion of the heart may be required.



As used herein, the term "part other than the myocardium of an adult" refers to any part, tissue, cell, or organ other than the myocardium of the terminally differentiated heart. Examples of such parts, tissues, cells, and organs include, but are not limited to, skeletal myoblasts, fibroblasts, synovial cells, stem cells, and the like. These cells have no marker characteristic to cells derived from the myocardium of the adult heart. Such a marker (hereinafter referred to as an "adult myocardial marker") may be in the form of a nucleic acid molecule (expression of mRNA), a protein, an extracellular matrix, a specific phenotype, a specific shape of a cell, or the like. Therefore, adult myocardial markers which are not specified herein may be used to identify a prosthetic tissue of the present invention as long as these markers can indicate cells derived from the myocardium of an adult. Representative examples of parts other than the myocardium of an adult include, but are not limited to, portions of the heart other than the adult myocardium, portions containing mesenchymal stem cells or cells derived therefrom, other tissues, other organs, myoblasts (e.g., skeletal myoblasts), fibroblasts, synovial cells, and the like. Therefore, by identifying a specific marker characteristic to parts other than the myocardium of an adult, the parts other than myocardium can be confirmed.

As used herein, the term "part other than the heart of an adult" refers to any part, tissue, cell, or organ other than the terminally differentiated heart. Examples of such parts, tissues, cells, and organs include, but are not limited to, skeletal myoblasts, fibroblasts, synovial cells, stem cells, and the like. These cells have no marker characteristic to cells derived from the adult heart. Such

a marker may be in the form of a nucleic acid molecule (expression of mRNA), a protein, an extracellular matrix, a specific phenotype, a specific shape of cell, or the like. Therefore, adult heart markers which are not specified herein  
5 may be used to identify a prosthetic tissue of the present invention as long as these markers can indicate cells derived from adult heart. Representative examples of parts other than the heart of an adult include, but are not limited to, parts containing mesenchymal stem cells or cells derived  
10 therefrom, other tissues, other organs, myoblasts (e.g., skeletal myoblasts), fibroblasts, synovial cells, and the like. Therefore, by identifying a specific marker characteristic to parts other than the heart, the parts other than the heart of an adult can be confirmed.

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A "part other than the myocardium of an adult" and a "part other than the heart of an adult" can be identified using markers characteristic to cells derived from the myocardium of an adult or the heart of an adult including  
20 skeletal myoblasts, fibroblasts, synovial cells, stem cells, or the like (hereinafter referred to as a "non-adult myocardial marker" or a "non-adult heart marker", respectively). If the marker is expressed by less than about 100%, preferably less than about 80%, more preferably less  
25 than about 50%, even more preferably less than about 25%, in some cases less than about 1%, the above-described parts can be identified. Examples of such markers include, but are not limited to, myosin heavy chain IIa, myosin heavy chain IIb, myosin heavy chain IIc (IIx), CD56, MyoD, Myf5,  
30 myogenin, and the like. Therefore, non-adult myocardial markers which are not specified herein may be used to identify a prosthetic tissue of the present invention as long as these markers can indicate cells derived from parts other than

the myocardium of an adult. Also, non-adult heart markers which are not specified herein may be used to identify a prosthetic tissue of the present invention as long as these markers can indicate cells derived from parts other than the heart of an adult.

Myosin heavy chain IIa (human: Accession No. NM\_017534; SEQ ID NOs. 1 and 2), myosin heavy chain IIb (human: Accession No. NM\_017533; SEQ ID NOs. 3 and 4), and myosin heavy chain IIc (IIx) (human: Accession No. NM\_005963; SEQ ID NOs. 5 and 6) are markers specific to myoblasts (Havenith M.G., Visser R., Schrijvers-van Schendel J.M., Bosman F.T., "Muscle Fiber Typing in Routinely Processed Skeletal Muscle With Monoclonal Antibodies", Histochemistry, 1990; 93(5):497-499). These markers can be confirmed mainly by observing the presence of proteins. An antibody against myosin heavy chain IIa, myosin heavy chain IIb, and myosin heavy chain IIc (IIx) is, for example, MY-32 available from Sigma. This antibody is specific to skeletal muscles and does not bind to myocardium (Webster C., Pavlath G.K., Parks D.R., Walsh F.S., Blau H.M., Exp. Cell. Res., 1988 Jan; 174(1):252-65; and Havenith M.G., Visser R., Schrijvers-van Schendel J.M., Bosman F.T., Muscle Fiber Typing in Routinely Processed Skeletal Muscle with Monoclonal Antibodies, Histochemistry, 1990, 93(5):497-499).

CD56 (human: Accession No. U63041; SEQ ID NOs. 7 and 8) is a marker specific to myoblasts. This marker can be confirmed mainly by observing the presence of mRNA.

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MyoD (human: Accession No. X56677; SEQ ID NOs. 9 and 10) is a marker specific to myoblasts. This marker can be confirmed mainly by observing the presence of mRNA.

Myf5 (human: Accession No. NM\_005593; SEQ ID NOs. 11 and 12) is a marker specific to myoblasts. This marker can be confirmed mainly by observing the presence of mRNA.

5

Myogenin (human: Accession No. BT007233; SEQ ID NOs. 13 and 14) is a marker specific to myoblasts. This marker can be confirmed mainly by observing the presence of mRNA.

10

In other embodiments, other markers specific to other tissues can be utilized. Examples of such markers include, but are not limited to, Oct-3/4, SSEA-1, Rex-1, Otx2, and the like for embryonic stem cells; VE-cadherin, Flk-1, Tie-1, PECAM1, vWF, c-kit, CD34, Thyl, Sca-1, and the like for endothelial cells; skeletal muscle  $\alpha$  actin in addition to the above-described markers for skeletal muscles; Nestin, Glu receptor, NMDA receptor, GFAP, neuregulin-1, and the like for nerve cells; c-kit, CD34, Thyl, Sca-1, GATA-1, GATA-2, FOG, and the like for hematopoietic cells.

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As used herein, the term "derived" in relation to cells means that the cells are separated, isolated, or extracted from a cell mass, tissue, or organ in which the cells have been originally present, or that the cells are induced from stem cells.

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As used herein, the term "applicable to heart" means that the heart applied has an ability to pulsate. A tissue applicable to heart has strength such that the tissue can withstand dilation and contraction of the pulsating heart. Here, applicability to the heart includes applicability to the myocardium. Applicability to heart may be determined

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by confirming that a recipient having an implanted graft survives.

As used herein, the term "three-dimensional  
5 structure" refers to an object which comprises cells having  
intracellular electrical connection and alignment and  
extends three-dimensionally. The term "three-dimensional  
structure" encompasses objects having any shape (e.g.,  
sheet-shape, etc.). A sheet-shaped structure comprises a  
10 single layer or a plurality of layers.

As used herein, the term "cell sheet" refers to a  
structure comprising a monolayer of cells. Such a cell sheet  
has at least a two-dimensional biological connection. The  
15 sheet having biological connection is characterized in that  
after the sheet is produced, the connection between cells  
is not substantially destroyed even when the sheet is handled  
singly. Such biological connection includes intracellular  
connection via an extracellular matrix.

20 As used herein, the term "biological connection" in  
relation to the relationship between cells means that there  
is certain interaction between the cells. Examples of such  
interaction includes, but are not limited to, interaction  
25 via biological molecules (e.g., extracellular matrix),  
interaction via signal transduction, electrical interaction  
(electrical connection, such as synchronization of  
electrical signals or the like), and the like. In order to  
confirm interaction, an assay appropriate to a characteristic  
30 of the interaction is employed. In order to confirm physical  
interaction via biological molecules, the strength of a  
prosthetic tissue, a three-dimensional structure, or the  
like is measured (e.g., a tensile test). In order to confirm

interaction via signal transduction, gene expression or the like is investigated. In order to confirm electrical interaction, the electric potential of a prosthetic tissue, a three-dimensional structure, or the like is measured to  
5 determine whether or not the electric potential is propagated with constant waves. Therefore, preferably, physical connection can be determined by observing whether or not the connection is established without a scaffold. In the present invention, it is typically sufficient that at least  
10 a two-dimensional biological connection is provided. In a preferred embodiment, it is advantageous that a three-dimensional biological connection is provided. In this case, a three-dimensional structure may be formed. Preferably, there is biological connection substantially  
15 uniformly in all directions in three-dimensional space. In another embodiment, the prosthetic tissue, a three-dimensional structure, and the like, which has substantially uniform two-dimensional biological connection and slightly weaker biological connection in the  
20 third dimension, may be employed.

A prosthetic tissue, three-dimensional structure, or the like of the present invention may be provided using known preparation methods, as a pharmaceutical product, or  
25 alternatively, as an animal drug, a quasi-drug, a marine drug, a cosmetic product, and the like.

Animals targeted by the present invention include any organism as long as it has organs (e.g., animals (e.g.,  
30 vertebrates, invertebrate)). Preferably, the animal is a vertebrate (e.g., Myxiniiformes, Petronyzoniformes, Chondrichthyes, Osteichthyes, amphibian, reptilian, avian, mammalian, etc.), more preferably mammalian (e.g.,

monotremata, marsupialia, edentate, dermoptera, chiroptera, carnivore, insectivore, proboscidea, perissodactyla, artiodactyla, tubulidentata, pholidota, sirenia, cetacean, primates, rodentia, lagomorpha, etc.). Illustrative  
5 examples of a subject include, but are not limited to, animals, such as cattle, pigs, horses, chickens, cats, dogs, and the like. More preferably, primates (e.g., chimpanzee, Japanese monkey, human, etc.) are used. Most preferably, a human is used. This is because there is limitation to  
10 implantation therapies.

When the present invention is used as a pharmaceutical agent, it may further comprise a pharmaceutically acceptable carrier or the like. A pharmaceutically acceptable carrier  
15 contained in a medicament of the present invention includes any material known in the art.

Examples of such a pharmaceutically acceptable carrier include, but are not limited to, antioxidants,  
20 preservatives, colorants, flavoring agents, diluents, emulsifiers, suspending agents, solvents, fillers, bulking agents, buffers, delivery vehicles, agricultural or pharmaceutical adjuvants, and the like.

25 The amount of a pharmaceutical agent (e.g., a prosthetic tissue, a pharmaceutical compound used in conjunction therewith, etc.) used in the treatment method of the present invention can be easily determined by those skilled in the art with reference to the purpose of use,  
30 a target disease (type, severity, and the like), the patient's age, weight, sex, and case history, the form or type of the cell, and the like. The frequency of the treatment method of the present invention applied to a subject (or patient)

is also determined by those skilled in the art with respect to the purpose of use, target disease (type, severity, and the like), the patient's age, weight, sex, and case history, the progression of the therapy, and the like. Examples of the frequency include once per day to several months (e.g., once per week to once per month). Preferably, administration is performed once per week to month with reference to the progression.

As used herein, the term "administer" in relation to a prosthetic tissue, three-dimensional structure, or the like of the present invention or a pharmaceutical agent comprising it, means that they are administered singly or in combination with other therapeutic agents. A prosthetic tissue of the present invention may be introduced into therapy sites (e.g., impaired heart, etc.) by the following methods, in the following forms, and in the following amounts. Examples of the introduction methods include, but are not limited to, direct attachment, suture after attachment, insertion, and the like. For example, a prosthetic tissue and a three-dimensional structure of the present invention may be applied by the above-described methods to an impaired site of ischemic myocardial tissue caused by myocardial infarct, angina pectoris, or the like. Combinations may be administered either concomitantly (e.g., as an admixture), separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously (e.g., a prosthetic tissue or the like is directly provided by operation, while other pharmaceutical agents are provided by intravenous injection). "Combination" administration



further includes the separate administration of one of the compounds or agents given first, followed by the second.

As used herein, the term "reinforcement" means that  
5 the function of a targeted part of an organism is improved.

As used herein, the term "instructions" describe a method of administering a medicament, a method for diagnosis, or the like of the present invention for persons who administer,  
10 or are administered, the medicament or the like or persons who diagnose or are diagnosed (e.g., physicians, patients, and the like). The instructions describe a statement indicating an appropriate method for administering a diagnostic, a medicament, or the like of the present invention.  
15 The instructions are prepared in accordance with a format defined by an authority of a country in which the present invention is practiced (e.g., Health, Labor and Welfare Ministry in Japan, Food and Drug Administration (FDA) in the U.S., and the like), explicitly describing that the  
20 instructions are approved by the authority. The instructions are so-called package insert and are typically provided in paper media. The instructions are not so limited and may be provided in the form of electronic media (e.g., web sites, electronic mails, and the like provided on the  
25 Internet).

As used herein, the term "stimulus responsive macromolecule" refers to a macromolecule which changes its shape and/or property in response to a stimulus, i.e., the  
30 changes occur between before and after the stimulus. Examples of such a stimulus include, but are not limited to, exposure to light, application of electric field, a change in temperature, a change in pH, addition of a chemical

substance, and the like. Examples of stimulus responsive macromolecules include, but are not limited to, poly(N-isopropylacrylamide), poly(N-isopropylacrylamide-acrylic acid) copolymer, 5 poly(N-isopropylacrylamide-methylmethacrylate) copolymer, poly(N-isopropylacrylamide-sodium acrylate) copolymer, poly(N-isopropylacrylamide-vinyl ferrocene) copolymer,  $\gamma$  ray-exposed poly(vinylmethylether) (PVME), poly(oxyethylene), a resin obtained by incorporating a 10 biological material (e.g., nucleic acid, etc.) into a macromolecule, and a gel obtained by cross-linking the above-described macromolecules with a cross-linking agent, and the like.

15 As used herein, the term "temperature responsive macromolecule" refers to a macromolecule which changes its shape and/or property in response to temperature. Examples of temperature responsive macromolecules include, but are not limited to, poly(N-isopropylacrylamide), 20 poly(N-isopropylacrylamide-acrylic acid) copolymer, poly(N-isopropylacrylamide-methylmethacrylate) copolymer, poly(N-isopropylacrylamide-sodium acrylate) copolymer, poly(N-isopropylacrylamide-vinyl ferrocene) copolymer,  $\gamma$  ray-exposed poly(vinylmethylether) (PVME), 25 poly(oxyethylene), and a gel obtained by cross-linking the above-described macromolecules with a cross-linking agent, and the like. Preferable examples of temperature responsive macromolecules include, but are not limited to, poly(N-isopropylacrylamide), 30 poly(N-isopropylacrylamide-methylmethacrylate) copolymer, poly(N-isopropylacrylamide-sodium acrylate) copolymer, and a gel obtained by cross-linking the above-described macromolecules with a cross-linking agent, and the like.

For example, a temperature responsive macromolecule used herein has an upper or lower critical solution temperature to water of from 0°C to 80°C. The present invention is not limited to this. The term "critical solution temperature" refers to a temperature threshold which changes a shape and/or property. Preferably, poly(N-isopropylacrylamide) may be herein used.

For example,  $\gamma$  ray-exposed polyvinylmethylether forms a hydrate at room temperature in aqueous solution which in turn swells. It is known that as the temperature is increased, the substance is dehydrated, so that the solution is contracted and turned into a heat-sensitive macromolecule gel. The PVME gel, which is uniform and transparent like a jelly, is turned cloudy (i.e., its transparency is changed) if it is heated. If the gel is provided with a porous structure or formed into fibers or particles, the gel can extend or contract at high speed. It is believed that a porous and fiber-like PVME gel can extend or contract for less than one second (see <http://www.aist.go.jp/NIMC/overview/v27-j.html>, Japanese Laid-Open Publication No. 2001-213992, and Japanese Laid-Open Publication No. 2001-131249). N-isopropylacrylamide gel (i.e., poly(N-isopropylacrylamide) is also known as a temperature responsive gel. If poly(N-isopropylacrylamide) is copolymerized with a hydrophobic monomer, the temperature which allows it to change its shape and/or property can be lowered. If poly(N-isopropylacrylamide) is copolymerized with a hydrophilic monomer, the temperature which allows it to change its shape and/or property can be raised. By utilizing this character, it is possible to prepare a filler responsive to a desired stimulus. Such a technique can be

applied to other temperature responsive macromolecules.

As used herein, the term "protein degrading enzyme" has the same meaning as commonly used in the art and refers to an enzyme which catalyzes the degradation of proteins. This enzyme is also referred to as "protease".

As used herein, the term "regular array film" refers to a film which has a structure in which elements are arrayed under a certain rule. Examples of the structure of such a film include, but are not limited to, honeycomb structure, line structure, dot structure, and the like. A prosthetic tissue of the present invention is preferably produced with such a regular array. The film can be made of a biodegradable material (e.g., poly-L-lactic acid (PLLA), etc.). In order to produce a stretchable film, poly( $\epsilon$ -caprolactone) (PCL) or the like can be used.

In cell engineering, tissue engineering, or the like, cell culture often requires base material for scaffolding cells. It is known that cell-to-cell interaction is affected by not only the chemical properties of cell surfaces but also the minute shape of cells. For the purpose of control of cellular functions, it is important to design both the chemical properties of material surfaces contacting cells and the minute structure of cells. It has been revealed that in porous films having honeycomb structure, the honeycomb pattern provides a cell adhesion surface and the porous structure provides access of cell supporting bases and supply routes of nutrients. Therefore, it is preferable to utilize honeycomb structure for the present invention.

The honeycomb structure film may be used as a base

to organize cells into a prosthetic organ or a prosthetic tissue, for example. Such a prosthetic organ or tissue or the like may be desired to be absorbed into the body. Therefore, the base is desirably absorbed into the body after  
5 a certain period of time. Conventional materials, which have honeycomb structure, stably maintain the structure for a period of time required for cell culture, and thereafter degrade, are described in, for example, Japanese Laid-Open Publication No. 2001-157574, Japanese Laid-Open  
10 Publication No. 2002-335949, and the like.

Japanese Laid-Open Publication No. 2001-157574 discloses a honeycomb structure and a film consisting of the honeycomb structure. The honeycomb structure is  
15 obtained as follows. A hydrophobic organic solvent solution of a polymer consisting of 50 to 99 w/w% of a biodegradable polymer and 50 to 1 w/w% of an amphipatic polymer is cast on a substrate in atmosphere having a relative humidity of 50 to 95%, followed by gradual evaporation of the organic  
20 solvent and concurrent dew condensation on the surface of the cast solution. Small dew drops caused by condensation are evaporated. As a result, a honeycomb structure is obtained. Japanese Laid-Open Publication No. 2002-335949 describes that the above-described film having a honeycomb  
25 structure can be used to form a three-dimensional aggregation of orderly organized cells which is similar to biological tissue.

A film for use in the present invention is produced  
30 as follows. A hydrophobic organic solvent solution of a single biodegradable and amphipatic polymer or a polymer mixture containing a biodegradable polymer and an amphipatic polymer is cast on a substrate, followed by evaporation of

the organic solvent and concurrent dew condensation on the surface of the cast solution. Small dew drops caused by condensation are evaporated.

5                   In the present invention, a single biodegradable and amphipatic polymer may be used, or alternatively, a polymer mixture containing a plurality of polymers including biodegradable polymer(s) and amphipatic polymer(s) may be used.

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                  As biodegradable polymers which can be used in the present invention, biodegradable aliphatic polyesters (e.g., poly(lactic acid), poly(hydroxybutyric acid), polycaprolactone, poly(ethylene adipate), poly(butylenes adipate), etc.), aliphatic polycarbonate (polybutylene carbonate, polyethylene carbonate, etc.), and the like are preferable in view of solubility to organic solvents. Particularly, poly(lactic acid) and polycaprolactone are desirable in view of availability, price, and the like.

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                  Amphipatic polymers which can be used in the present invention are preferably nontoxic in view of use of them as a base material for cell culture. Examples of such amphipatic polymers include, but are not limited to, polyethylene glycol/polypropylene glycol block copolymer; an amphipatic polymer having an acrylamide polymer as a main chain (backbone structure), a dodecy group as a hydrophobic side chain, and a lactose group or a carboxyl group as a hydrophilic side chain; an ionic complex of an anionic macromolecule (e.g., heparin, dextran sulfate, nucleic acid (e.g., DNA, RNA, etc.), etc.) and a long chain alkylammonium salt; an amphipatic polymer having a water-soluble protein (e.g., gelatin, collagen, albumin, etc.) as a hydrophilic

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group; and the like. Examples of a single biodegradable and amphipatic polymer include, but are not limited to, polylactic acid/polyethylene glycol block copolymer, poly( $\epsilon$ -caprolactone)/polyethylene glycol block copolymer, 5 poly(malic acid)/(an alkyl ester of polymalic acid) block copolymer, and the like.

As used herein, the term "impaired heart" and "impaired myocardium" refer to a heart and a myocardium, 10 respectively, having an impairment including, but being limited to, ischemic impairments and the like. Examples of ischemic impairments include, but are not limited to, myocardial infarct, angina pectoris, and the like.

15 As used herein, the term "three-dimensional promoting agent" refers to an agent which promotes biological connection in a third dimension after a group of cells are prepared. Examples of such an agent representatively include agents capable of promoting the secretion of a 20 cellularmatrix. Examples of a three-dimensional promoting agent include, but are not limited to, ascorbic acid or a derivative thereof (e.g., ascorbic acid 2-phosphate, ascorbic acid 1-phosphate, sodium L-ascorbate, etc.), and the like. Preferably, a three-dimensional promoting agent 25 may be preferably a component of an extracellular matrix of a part targeted by application and/or a component(s) capable of promoting the secretion of an extracellularmatrix in an amount similar thereto. When a three-dimensional promoting agent comprises a plurality of components, the 30 components may be components of an extracellular matrix of a part targeted by application and/or components capable of promoting the secretion of an extracellular matrix in an amount similar thereto.

As used herein, the term "ascorbic acid or a derivative thereof" includes ascorbic acid and an analog thereto (e.g., ascorbic acid 2-phosphate, ascorbic acid  
5 1-phosphate, etc.), and a salt thereof (e.g., sodium salt, magnesium salt, etc.).

(Description of the Preferred Embodiments)

Hereinafter, preferred embodiments of the present  
10 invention will be described. The following embodiments are provided for a better understanding of the present invention and the scope of the present invention should not be limited to the following description. It will be clearly appreciated by those skilled in the art that variations and modifications  
15 can be made without departing from the scope of the present invention with reference to the specification.

According to one aspect of the present invention, a three-dimensional structure applicable to heart, which  
20 comprises a cell derived from a part other than the myocardium of an adult, is provided. Conventional three-dimensional structures applicable to heart comprise a cell derived from the myocardium of an adult, and have a small size and poor performance. The present invention is the first in the world  
25 to provide a three-dimensional structure applicable to heart, which comprises a cell derived from a part other than the myocardium of an adult (i.e., non-embryo), by culturing the cell derived from the part other than the myocardium of an adult under specific conditions (e.g., in the presence of  
30 a three-dimensional promoting agent, etc.). The three-dimensional structure comprises a cell, preferably substantially a cell and a component derived from the cell (e.g., an extracellular matrix, etc.). Thus, in a preferred



embodiment, the three-dimensional structure of the present invention comprises a biological material, and therefore, advantageously overcomes drawbacks due to a scaffold (e.g., poor biocompatibility, immunogenicity, etc.) as compared to conventional structures having a scaffold.

In one preferred embodiment, a cell contained in the three-dimensional structure of the present invention may include both a stem cell and a differentiated cell. In a preferred embodiment, a cell contained in the three-dimensional structure of the present invention is a mesenchymal cell. Though not wishing to be bound by any theory, the reason a mesenchymal cell is preferable is that the mesenchymal cell itself is excellently compatible to the heart, and may have an ability to differentiate into the heart tissue.

The above-described mesenchymal cell may be a mesenchymal stem cell or a differentiated mesenchymal cell.

Examples of a mesenchymal cell for use in the present invention include, but are not limited to, bone marrow, fat cells, synovial cells, and the like.

In a more preferred embodiment, a cell used in the present invention is preferably derived from a myoblast. Conventionally, it was not possible to expect that a three-dimensional structure comprising a myoblast is applicable to heart. This finding is an astonishing effect. The reason myoblasts are preferable is that, for example, the supply source thereof is abundant. The reason is not limited to this.

In a more preferred embodiment, a cell used in the present invention is a skeletal myoblast. Skeletal myoblasts are abundant, and therefore, are preferable as an easily available supply source. In addition, the present invention revealed for the first time the possibility that skeletal myoblasts can be implanted into a heart. Thus, skeletal myoblasts can be used in actual medical practice.

Thus, by appropriately culturing cells derived from parts other than the autologous myocardium, a defective heart can be repaired without implantation surgery.

In another embodiment, a cell used in the present invention may be a fibroblast. This is because fibroblasts can provide biological connection three-dimensionally in a three-dimensional structure. A three-dimensional structure comprising a fibroblast can be used in heart applications. Alternatively, such a three-dimensional structure comprising a fibroblast can be used for reinforcement in addition to heart applications.

In another embodiment, a cell used in the present invention may be a synovial cell. This is because synovial cells can provide biological connection three-dimensionally in a three-dimensional structure. A three-dimensional structure comprising a synovial cell can be used in heart applications. Alternatively, such a three-dimensional structure comprising a synovial cell can be used for reinforcement in addition to heart applications.

In another embodiment, a cell used in the present invention is derived from a stem cell. This is because a three-dimensional structure comprising a cell derived from

a stem cell can utilize a cell which is differentiated in a desired direction. Therefore, when the structure is applied to a heart, a stem cell which has been differentiated into a heart cell may be preferable. An exemplary method  
5 for differentiation may employ LIF. The present invention is not limited to this.

In a preferred embodiment, a cell used in the present invention is advantageously a cell derived from a subject  
10 to which a three-dimensional structure is applied. In this case, a cell used herein is also referred to as an autologous cell. By using autologous cells, immune rejection reactions can be prevented or reduced.

15 In another embodiment, a cell used in the present invention may be a cell not derived from a subject to which a three-dimensional structure is applied. In this case, a measure for preventing immune rejection reactions is preferably taken.

20 In another embodiment, a cell used in the three-dimensional structure of the present invention expresses at least one non-adult heart marker selected from the group consisting of myosin heavy chain IIa, myosin heavy  
25 chain IIb, myosin heavy chain IIc(IIX), CD56, MyoD, Myf5, and myogenin. By utilizing such a non-adult heart marker, it is possible to confirm that the cell used in the three-dimensional structure is a non-adult heart-derived cell. The three-dimensional structure of the present  
30 invention can avoid the use of the heart of an adult to significantly increase the potential of heart therapies.

The above-described non-adult heart marker is

expressed at a level which is typically possessed by a non-adult heart or a tissue thereof. For example, such a level may be at least about 50% of the naturally-occurring level of a non-adult heart or a tissue thereof, preferably at least about 60%, more preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, and still even more preferably at least about 100%. Examples of a technique for determining the level include, but are not limited to, PCR, blotting for determining the expression level of mRNA (e.g., Northern blotting, etc.), blotting for determining the expression level of a protein (e.g., Western blotting, etc.), and the like. PCR is utilized as follows. A specific primer is selected and designed from the above-described non-adult heart markers by using a method well known in the art (e.g., a commercially available PCR primer design device is used). Samples containing mRNA are extracted from a tissue or cell of interest. cDNA is prepared from mRNA using techniques well known in the art. cDNA is subjected to PCR cycles which allow detection of specific expression. The amplified products are subjected to, for example, electrophoresis, followed by staining. As a result, the expression level can be determined. Northern blotting is utilized as follows. The whole or a part of the nucleic acid sequence of the above-described non-adult heart marker is prepared as a probe (particularly, a sequence capable of specific detection). Samples containing mRNA are extracted from a tissue or cell of interest, followed by separation using electrophoresis. The above-described probe is used to detect expression. When a marker is involved in protein expression, antibodies specific to the protein are prepared. These antibodies are used in Western blotting to detect expression by utilizing antigen-antibody reactions.

In another embodiment, a cell used in the three-dimensional structure of the present invention contains substantially no adult heart marker. Based on the  
5 fact that the cell contains substantially no adult heart marker, it is possible to confirm that the three-dimensional structure uses a non-adult heart-derived cell. The three-dimensional structure of the present invention can avoid the use of the heart of an adult to significantly increase  
10 the potential of heart therapies.

The above-described adult heart marker is expressed at less than a level which is typically possessed by an adult heart or a tissue thereof. For example, such a level may  
15 be less than about 100% of the naturally-occurring level of an adult heart or a tissue thereof, preferably less than about 80%, more preferably less than about 50%, even more preferably less than about 20%, still more preferably less than about 10%, and still even more preferably less than  
20 about 5%.

In a preferred embodiment, a cell used in the three-dimensional structure of the present invention contains substantially no adult heart markers. By  
25 confirming the absence of all adult heart markers, it is possible to more reliably confirm that the structure is not an adult heart. Note that not all adult heart markers are always checked.

30 Preferably, a cell used in the three-dimensional structure of the present invention is preferably a cell other than heart cells. Cells other than cardiomyocytes can be used in the present invention. However, cells derived from

a heart have a limited supply source, and cells derived from an autologous heart are substantially not available. Therefore, the use of cells not derived from a heart is preferable.

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The three-dimensional structure of the present invention is typically applicable to heart, and may be applied to other organs. Preferably, the three-dimensional structure of the present invention may be applied to a myocardium.

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In one embodiment, the three-dimensional structure of the present invention comprises a cell sheet having at least one layer. In a certain embodiment, the three-dimensional structure of the present invention comprises a cell sheet having only one layer. By providing a cell sheet, the intact or nonporous nature of the three-dimensional structure of the present invention is secured. Therefore, the three-dimensional structure of the present invention comprising a cell sheet having at least one layer is useful in applications in which an injured site is covered.

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In a preferred embodiment, the three-dimensional structure of the present invention comprises a cell sheet having a plurality of layers. Preferably, it is advantageous that a plurality of layers in the cell sheet advantageously biologically connect one another. In this case, biological connection is preferably physical connection via an extracellular matrix, or electrical connection (e.g., pulsation, etc.). The connection varies depending on the desired site. When the three-dimensional structure of the present invention is intended to be implanted into a heart,

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the biological connection typically includes electrical connection. Alternatively, the biological connection may be connection without a scaffold.

5           The three-dimensional structure of the present invention may be provided as a pharmaceutical agent. Alternatively, the three-dimensional structure of the present invention may be prepared by a medical practitioner or the like at an actual site. Alternatively, after a medical  
10 practitioner prepares cells, a third party may culture the cells and prepare a three-dimensional structure comprising the cells, and employ it for surgery. In this case, cell culture can be carried out by those skilled in the art as well as medical practitioners. Therefore, those skilled in  
15 the art can determine culture conditions depending on the type of cells and the target implantation site in accordance with the disclosure of the present specification.

          In another aspect of the present invention, a method  
20 is provided for producing a three-dimensional structure applicable to adult heart, which comprises a cell derived from a part other than the heart. The method comprises the steps of: a) culturing a cell derived from a part other than the myocardium of an adult on a support comprising a  
25 temperature responsive macromolecule; b) setting a culture temperature outside a critical solution temperature range of the temperature responsive macromolecule (above the upper limit, if any; or below the lower limit, if any); and  
30 c) detaching the cultured cell as a three-dimensional structure. In this case, the cell derived from a part other than a myocardium may be a cell derived from a part other than a heart, including, for example, mesenchymal cells (e.g., myoblasts, skeletal myoblasts, synovial cells, fibroblasts,

etc.), and the like. Preferably, the upper limit or lower critical solution temperature to water is from 0°C to 80°C. A temperature responsive macromolecule having the above-described critical solution temperature is preferably grafted in the support.

In a preferred embodiment of the present invention, in the method for producing a three-dimensional structure, it is preferable that the structure is not treated with a protein degrading enzyme in or before the detaching step. In conventional methods for preparing cell sheets or the like, treatment using a protein degrading enzyme is performed so as to facilitate detachment. In this case, however, the cell sheet is injured, and therefore, cannot be used as a three-dimensional structure. In the method of the present invention, treatment using a protein degrading enzyme can be omitted, whereby an intact three-dimensional structure can be achieved.

In a preferred embodiment, the temperature responsive macromolecule is poly(N-isopropylacrylamide). Poly(N-isopropylacrylamide) has a lower limit critical solution temperature of a little more than 20°C. In this case, therefore, by changing culture medium from a typical culture temperature (e.g., about 37°C) to about 20°C, it is possible to easily prepare a three-dimensional structure. Thereby, a three-dimensional structure applicable to implantation surgery, which comprises a cell not derived from the myocardium of an adult, can be provided. As a result, a technique substantially other than organ transplantation can be provided to a number of diseases which can be treated only by conventional heart transplantation (e.g., dilated cardiomyopathy, etc.). That is, a therapy which cannot be



achieved by conventional techniques can be provided by the present invention.

5 In a more preferred embodiment, the three-dimensional structure of the present invention preferably comprises a three-dimensional organization promoting agent when cell culture is conducted. Such a three-dimensional organization promoting agent may be ascorbic acid or a derivative thereof.

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In another aspect, the prosthetic tissue and three-dimensional structure of the present invention is free from injury caused by a protein degrading enzyme, such as, representatively, dispase, trypsin, or the like, during culture. Therefore, the prosthetic tissue and three-dimensional structure, which is detached from the base material, can be recovered as a cell mass holding proteins between cells (e.g., an extracellular matrix) and having a certain level of strength. The prosthetic tissue and three-dimensional structure also retain intact functions, such as a contraction/relaxation function, intracellular electrical connection, alignment, and the like which are specific to cardiomyocytes. In addition, the three-dimensional structure may have several characteristic, biological tissue-like, cell alignments (e.g., formation of a membrane consisting of connective tissue, formation of a lumen consisting of blood vessel endothelial cells, etc.). When typical protein degrading enzymes (e.g., trypsin, etc.) are used to detach the three-dimensional structure or prosthetic tissue, substantially no desmosome structure between cells, basement membrane-like proteins between cells and base materials, or the like are retained, so that cells are individually separated. Among these

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protein degrading enzymes, dispase destroys basement membrane-like proteins between cells and base materials substantially completely, but not necessarily desmosome structure. The desmosome structure can be retained if the  
5 temperature is from 10°C to 60°C. In this case, however, the resultant three-dimensional structure or prosthetic tissue has weak strength. In contrast, the three-dimensional structure or prosthetic tissue of the present invention can retain 80% or more of each of the  
10 desmosome structure and the basement membrane-like protein, resulting in the above-described various effects. In the cell culture support, a temperature responsive polymer for use in coating the base material has an upper or lower limit critical solution temperature of from 0°C to 80°C in aqueous  
15 solution, and preferably from 20°C to 50°C. If the upper or lower limit critical solution temperature exceeds 80°C, cells are likely to be killed. If the upper or lower limit critical solution temperature is lower than 0°C, the cell growth rate is extremely low or cells are killed.

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The temperature responsive polymer used in the present invention may be either a homopolymer or a copolymer. An example of such a polymer is a polymer described in, for example, Japanese Laid-Open Publication No. 2-211865, in  
25 addition to those described above. Specifically, for example, such a temperature responsive polymer is obtained by homo- or co-polymerization of the following monomers. Examples of usable monomers include, but are not limited to, (meth)acrylamide compounds, N-(or N,N-di)alkyl  
30 substituted (meth)acrylamide derivatives, or vinyl ether derivatives. In the case of copolymers, any two or more of these monomers can be used. Further, copolymers comprising monomers other than the above-described monomers, copolymers

obtained by graft- or co-polymerization of polymers, or a mixture of homopolymers and copolymers may be employed. Furthermore, polymers may be cross-linked to an extent such that the inherent properties of the polymer are not impaired.

5 Examples of the base material to be coated include, but are not limited to, materials typically used in cell culture (e.g., glass, modified glass, polystyrene, polymethylmethacrylate, etc.), substantially all materials capable of being generally shaped (e.g., macromolecule

10 compounds in addition to those described above, ceramics, etc.).

A method for coating a support with a temperature responsive polymer is not particularly limited and may be

15 performed in accordance with, for example, Japanese Laid-Open Publication No. 2-211865. Specifically, coating may be performed by subjecting a base material and the above-described monomer or polymer to electron beam irradiation (EB),  $\gamma$  ray irradiation, ultraviolet irradiation,

20 plasma treatment, corona treatment, or organic polymerization reaction, or physical adsorption or the like (e.g., application, crossing, etc.).

In the present invention, cell culture is conducted

25 on the above-described cell culture support (e.g., a cell culture dish, etc.). When the above-described polymer covering the surface of the base material has an upper limit critical solution temperature, the temperature of the medium is set to be equal to or lower than the upper limit critical

30 solution temperature. When the above-described polymer covering the surface of the base material has a lower limit critical solution temperature, the temperature of the medium is set to be equal to or higher than the lower limit critical

solution temperature. However, a low temperature range such that cultured cells cannot grow and a high temperature range such that cultured cells are killed is inappropriate. Culture conditions other than temperature may be those well  
5 known in the art and are not particularly limited. For example, the medium may be supplemented with known fetal calf serum (FCS) or the like, or alternatively, serum-free medium without supplement of such a serum may be employed.

10 In the method of the present invention, the period of time required for culture may be determined depending on the application of the prosthetic tissue or three-dimensional structure. In order to detach and recover the cultured prosthetic tissue or three-dimensional  
15 structure from the support material, the temperature of the support material having attached cells is increased on or above the upper limit critical solution temperature of a polymer covering the support base material, or is decreased on or below the lower limit critical solution temperature.  
20 In this case, the cultured prosthetic tissue or three-dimensional structure is detached directly, or optionally with macromolecular membrane being attached thereto. Note that the prosthetic tissue or three-dimensional structure may be detached in culture medium  
25 in which cells have been cultured, or alternatively, in other isotonic solutions. Such solutions may be selected depending on the purpose. Examples of the macromolecular membrane, which is optionally attached to the cell sheet or three-dimensional structure, include, but are not limited to, hydrophilized polyvinylidene difluoride (PVDF),  
30 polypropylene, polyethylene, cellulose and derivatives thereof, chitin, chitosan, collagen, paper (e.g., Japan paper, etc.), urethane, net-like or stockinette -like

macromolecular materials (e.g., spandex, etc.), and the like. When a net-like or stockinette-like macromolecular material is employed, the prosthetic tissue or three-dimensional structure has a higher degree of freedom, so that the contraction/relaxation function thereof can be increased. 5 A method for producing the prosthetic tissue or three-dimensional structure comprising cells of the present invention is not particularly limited. For example, the prosthetic tissue or three-dimensional structure of the 10 present invention can be produced by utilizing the above-described cultured cell sheet attached to a macromolecular membrane.

In order to detach and recover the prosthetic tissue or three-dimensional structure with a high yield from the cell culture support, the cell culture support is tapped or shaken, or the medium is stirred with a pipette. These procedures may be performed singly or in combination. In addition, the prosthetic tissue or three-dimensional 20 structure may be optionally rinsed with isotonic solution or the like before detaching and recovering. By stretching the prosthetic tissue or three-dimensional structure in a specific direction after being detached from the base material, the cell sheet or three-dimensional structure is 25 provided with alignment. Stretching may be performed by using a tensile device (e.g., Tensilon, etc.), or simply forceps, or the like. A stretching method is not particularly limited. By providing alignment, it is possible to confer directionality to the motion of the cell sheet or 30 three-dimensional structure itself. Therefore, for example, it is possible to allow the prosthetic tissue or three-dimensional structure to move in accordance with the motion of a specific organ. The prosthetic tissue or

three-dimensional structure can be efficiently applied to organs.

The thus-obtained prosthetic tissue or  
5 three-dimensional structure cannot be obtained by  
conventional techniques. The prosthetic tissue or  
three-dimensional structure retains the basement membrane  
which in conventional techniques is ruptured. Therefore,  
the prosthetic tissue or three-dimensional structure can  
10 be satisfactorily accepted by the surrounding tissue and  
can pulsate *in situ* when it is buried in any part of an organism  
(e.g., heart, bone, muscle, arm, shoulder, foot, and other  
organs). Though not wishing to be bound by any theory, the  
reason is believed to be as follows. The prosthetic tissue  
15 or three-dimensional structure buried within an organism  
is accepted by a biological tissue, and is contracted or  
relaxed. In this case, the prosthetic tissue or  
three-dimensional structure is in the low oxygen state. To  
compensate the state, blood vessel endothelial cells  
20 aggressively enter the prosthetic tissue or  
three-dimensional structure from the surrounding biological  
tissue. As a result, blood vessels are formed, so that  
sufficient oxygen and nutrients can be supplied via blood.  
Thus, the prosthetic tissue or three-dimensional structure  
25 buried within an organism can form a functional tissue within  
the organism. Such a prosthetic tissue or three-dimensional  
structure is strongly expected to be used in clinical  
applications, such as implantation and the like.  
Specifically, the prosthetic tissue or three-dimensional  
30 structure of the present invention can be used as a therapeutic  
instrument for heart diseases (e.g., myocardial infarct,  
etc.). In this case, for example, the prosthetic tissue or  
three-dimensional structure is implanted to a site of a heart

which has a weak contraction force. Alternatively, the prosthetic tissue or three-dimensional structure may be applied around a blood vessel to improve circulation. For example, the prosthetic tissue or three-dimensional structure is useful as a therapeutic instrument for severe Raynaud's disease, a severe stiff shoulder, the dysfunction of the aorta, and the like. Note that a cell culture support for use in the present invention can be repeatedly used.

(Preparation of prosthetic tissue using three-dimensional promoting agent)

In another aspect, the present invention provides a method for producing a prosthetic tissue. The method for producing a prosthetic tissue comprises the steps of: A) providing a cell; B) placing the cell in a container containing a cell culture medium including a three-dimensional promoting agent, wherein the container has a base with an area sufficient to accommodate a desired size of the prosthetic tissue; and C) culturing the cell in the container for a period of time sufficient to form the prosthetic tissue having the desired size.

The above-described cell may be any cell. A method for providing a cell is well known in the art. For example, a tissue is extracted and cells are isolated from the tissue. Alternatively, cells are isolated from body fluid containing blood cells or the like. Alternatively, a cell line is prepared in an artificial culture. The present invention is not limited to this.

The method for producing a prosthetic tissue of the present invention employs a cell culture medium containing a three-dimensional promoting agent. Examples of such a

three-dimensional promoting agent include, but are not limited to, ascorbic acid or a derivative thereof, ascorbic acid 1-phosphate, ascorbic acid 2-phosphate, L-ascorbic acid, and the like.

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The cell culture medium used in the present invention may be any medium which allows a cell of interest to grow. Examples of such a medium include, but are not limited to, DMEM, MEM, F12, DME, RPMI1640, MCDB104, 199, MCDB153, L15, SkBM, Basal medium, and the like which are supplemented with glucose, FCS (fetal calf serum), antibiotics (penicillin, streptomycin, etc.) as appropriate.

The container used in the present invention may be any container typically used in the art which has a base with an area sufficient to accommodate a desired size of the prosthetic tissue. Examples of such a container include, but are not limited to, petri dishes, flasks, mold containers, and the like, and preferably containers having a large area of the base (e.g., at least 1 cm<sup>2</sup>). The material of the container may be any material and include, but are not limited to, glass, plastic (e.g., polystyrene, polycarbonate, etc.), silicone, and the like.

In a preferred embodiment, the three-dimensional promoting agent used in the method for producing a prosthetic tissue of the present invention includes ascorbic acid 2-phosphate. Conventionally, it is known that ascorbic acid is used for cell culture. However, there was no report that ascorbic acid 2-phosphate is intentionally used for formation of tissue. In the present invention, by adding a certain amount of ascorbic acid 2-phosphate, it is possible to prevent an extracellular matrix from being produced in an excessively



large amount, the composition has an implantable level of extracellular matrices, and therefore, the ratio of the cell to the extracellular matrix results in an implantable level of strength and the like. These effects are unexpected.

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In a preferred embodiment, ascorbic acid 2-phosphate used in the present invention typically has a concentration of at least 0.01 mM, preferably at least 0.05 mM, more preferably at least 0.1 mM, even more preferably at least 10 0.2 mM, still more preferably at least 0.5 mM, and still even more preferably 1.0 mM.

Alternatively, ascorbic acid 2-phosphate used in the present invention may be used in conjunction with ascorbic 15 acid 1-phosphate. In this case, ascorbic acid 1-phosphate and ascorbic acid 2-phosphate may be preferably used at a specific ratio. Such a preferable ratio is, for example, in the range of from 1:10 to 10:1. Alternatively, the preferable ratio is such that the molar amount of ascorbic 20 acid 1-phosphate is smaller than the molar amount of ascorbic acid 2-phosphate.

In another embodiment, the three-dimensional promoting agent used in the present invention includes 25 ascorbic acid 2-phosphate. However, there was no report that ascorbic acid 2-phosphate is explicitly used for formation of a tissue. In the present invention, by adding a certain amount of ascorbic acid 2-phosphate, it is possible to prevent an extracellular matrix from being produced in an excessively 30 large amount, the composition has an implantable level of extracellular matrices, and therefore, the ratio of cells to the extracellular matrix results in an implantable level of strength and the like. These effects are unexpected.

In a preferred embodiment, when the molar amount of ascorbic acid 1-phosphate is smaller than the molar amount of ascorbic acid 2-phosphate, ascorbic acid 2-phosphate used  
5 in the present invention is typically present at a concentration of at least 0.01 mM, preferably at least 0.05 mM, more preferably at least 0.1 mM, even more preferably at least 0.2 mM, and still more preferably at least 0.5 mM, and still even more preferably 1.0 mM.

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In a certain preferred embodiment, the three-dimensional promoting agent of the present invention includes ascorbic acid 1-phosphate or a salt thereof, ascorbic acid 2-phosphate or a salt thereof, and L-ascorbic  
15 acid or a salt thereof.

The container used in a prosthetic tissue production method of the present invention is preferably coated with a temperature responsive macromolecule. In another  
20 preferred embodiment, the container is preferably provided with a scaffold having a honeycomb structure. The present invention is not limited to this. Use of a honeycomb structure is described in, for example, Tanaka K. et al, "Atarashii Baiomedikaru Intafesu [New Biomedical  
25 Interface]", Kagaku Kogyo [Chemical Industry], December 2002, 901-906, Kagaku Kogyo Sha.

The temperature responsive macromolecule which may be used in the prosthetic tissue production method of the present invention, includes poly(N-isopropylacrylamide).  
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In a preferred embodiment, after the culturing step, the prosthetic tissue production method of the present

invention further comprises, D) detaching the prosthetic tissue and allowing the prosthetic tissue to perform self contraction. The detachment can be accelerated by applying a physical stimulus (e.g., applying a physical stimulus to a corner of the container, etc.). When a temperature responsive macromolecule is used, the detachment can be accelerated by changing the environment to a temperature higher or lower than the critical solution temperature of the temperature responsive macromolecule. Self-contraction naturally takes place after the detachment. By self-contraction, biological connection is accelerated particularly in the third dimension (the direction perpendicular to the two-dimensional directions in the case of tissue on a sheet). Therefore, a prosthetic tissue of the present invention may have a three-dimensional structure.

In a prosthetic tissue production method of the present invention, the sufficient time preferably means at least 3 days and may be more than or less than 3 days, though it varies depending on the application of a prosthetic tissue of interest. By 3-day culture, it is possible to prepare a graft which can be applied at least to the reinforcement of a heart.

In another aspect, the present invention provides a functional prosthetic tissue. The functional prosthetic tissue of the present invention is herein an implantable prosthetic tissue. Attempts have been heretofore made to produce prosthetic tissues by cell culture. However, there were no prosthetic tissues suitable for implantation in terms of size, strength, physical injuries when it is detached, or the like. The present invention provides a tissue culture method in which cells are cultured in the presence of a

three-dimensional promoting agent as described above, so that there is no problem in terms of size, strength, and the like and there is no difficulty in detaching tissues. An implantable prosthetic tissue is provided only after such  
5 a tissue culture method is achieved. For diseases which are conventionally treated only by organ transplantation (e.g., refractory heart diseases (myocarditis, hypertrophic cardiomyopathy, dilated phase hypertrophic cardiomyopathy, dilated cardiomyopathy, etc.)), an alternative therapy  
10 having general versatility is provided, which is of immeasurable usefulness.

A "disease" targeted by the present invention may be any heart disease in which tissue is injured. Examples  
15 of such a heart disease include, but are not limited to, heart failure, myocardial infarct, cardiomyopathy, and the like. A combined therapy of the present invention may be applied to organs in addition to the heart as long as it aims the regeneration of injured tissues. In a specific  
20 embodiment, a disease targeted by a method of the present invention is refractory heart failure.

The term "heart failure" refers to the inability of the heart to circulate blood in a required quantity and quality  
25 to organs in the entire body due to an impairment of the heart itself, such as failure of cardiac functions, failure of circulatory functions, a reduction in contractile power, or the like. Heart failure is a terminal symptom of heart diseases, such as myocardial infarct, cardiomyopathy, and  
30 the like. Severe heart failure means that the state of the heart is severe and is also referred to as terminal heart failure.

The term "refractory heart failure" refers to heart failure which is resistant to therapy and is difficult to ameliorate by medical therapy and drug therapy. The term "refractory heart failure" is substantially synonymically referred to as chronic heart failure or terminal heart failure. Refractory heart failure cannot be controlled by triple therapy using a typical Digitalis drug, diuretic, ACE inhibitor, and the like, or a drug therapy supplemented with a  $\beta$ -blocker. These therapies require a mechanical aid for circulation, such as IABP (intra-aortic balloon pumping), PCPS (percutaneous cardiopulmonary support), or the like, or alternatively, heart transplantation. Therefore, there was a demand for the development of a simple and radical therapy. Particularly, heart transplantation has a serious donor shortage problem. When heart transplantation cannot be used (e.g., elderly, patients in need of dialysis, etc.), refractory heart failure poses serious problems. Therefore, there is a keen demand for an alternative to heart transplantation therapy.

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The term "myocardial infarct" refers to a disease in which ischemic necrosis occurs in a perfusion area associated with highly developed constriction or occlusion caused by various lesions of the coronary artery. The severity of myocardial infarct is divided into classes in various manners. Classification may be based on, for example, progress over time; morphology (e.g., the range, site, necrosis size, or the like within the myocardium); the necrosis form of a myocardium; the reconstruction of a ventricle after infarction; the dynamics of blood circulation (associated with therapy, prognosis, etc.); clinical severity; and the like. Myocardial infarct having a high level of severity is particularly called severe myocardial

30

infarct.

The term "cardiomyopathy" is a generic term for diseases caused by organic and functional abnormality in  
5 amyocardium, which are divided into secondary cardiomyopathy following a basic disease (e.g., hypertension, dysbolism, ischemia, etc.), and spontaneous cardiomyopathy which develops without an apparent basic disease. As a  
10 pathological change, myocardial hypertrophy, formation of fibrous tissue, degeneration, or the like is observed.

The term "dilated cardiomyopathy" refers to a functional failure of the left ventricle associated with the deflation thereof, and is also referred to as "congestive  
15 cardiomyopathy". The term "dilated cardiomyopathy" may be abbreviated as "DCM". Dilated cardiomyopathy is associated with contraction failure, leading to chronic heart failure. Examples of a cause of dilated cardiomyopathy include various things, such as viral infection, gene mutation, and the like.  
20 Generally, dilated cardiomyopathy does not include specific myocardial diseases (conventionally referred to as secondary myocardial diseases), such as ischemic cardiomyopathy caused by an unambiguous other cause, myocardial diseases associated with dysbolism or the like. However, these specific  
25 myocardial diseases are also included within the scope of the present invention as long as the present invention has a therapeutic effect thereon. Most patients have a reduction in contractile power in the entire myocardium. It is also said that abnormality may occur in the motion of an isolated  
30 portion of a wall. Typically, dilated cardiomyopathy has heart failure symptoms associated with congestion and sometimes has malaise due to low cardiac output. Dilated cardiomyopathy has no known cause and is an idiopathic

myocardial disease. The pathology of dilated cardiomyopathy mainly includes a reduction in contractile power of a myocardium, which leads to the dilation of the left ventricular cavity. The pathology also includes a  
5 reduction in blood amount, an increase in left ventricular diastolic pressure, and the like. Dilated cardiomyopathy has acute or insidious onset and refractory heart failure at its end stage. Pathologically or histologically, the degeneration, formation of fibrous tissue, or atrophy of  
10 myocardial tissue is diffusely or locally observed. Residual cardiomyocytes often cause hypertrophy. In addition to heart failure, severe arrhythmia or thromboembolism occurs, resulting in a very poor prognosis. Echocardiogram is particularly useful for diagnosis of  
15 dilated cardiomyopathy, which demonstrates a diffuse reduction in wall motion, the thinning of a ventricular wall, and the dilation of a ventricular cavity. In addition, coronary angiography (coronary arterial lesion) and myocardial biopsy can be conducted to achieve a more reliable  
20 diagnosis. Therefore, in the present invention, a test technique well known in the art, such as heart ultrasonography, a heart catheter test method, a nuclear medical test method (myocardial scintigraphy), myocardial biopsy, or the like, can be used to confirm an amelioration in dilated  
25 cardiomyopathy.

Conventionally, for dilated cardiomyopathy, a drug therapy using an ACE inhibitor, a diuretic, a  $\beta$ -blocker, a cardiotonic drug, or the like; guidance in regulating the  
30 intake of salt and water and exercise; and the like, are conducted. However, none of them cure the disease itself. For arrhythmia, an antiarrhythmic agent, such as amiodarone or the like, may be administered, which is however only a

symptomatic treatment. For thrombi and emboli, an anticoagulant, such as warfarin or the like, is used, which is however only a symptomatic treatment. As a surgical treatment, a pacemaker, a buried defibrillator, an assisted  
5 circulation device (bypass), heart transplantation, or the like is used. However, these treatments other than heart transplantation cannot be said to eradicate the disease. At present, serious donor shortage limits heart transplantation. A therapeutic technique of the present  
10 invention is effective for dilated cardiomyopathy and the like and provides an advantageous therapeutic effect.

The term "hypertrophic cardiomyopathy" (HCM) refers to a type of cardiomyopathy whose main symptom is a reduction  
15 in diastolic compliance due to an abnormal enlargement of a myocardium and left ventricle hypertrophy. The contractile function of the heart is typically retained. The 5-year and 10-year survival rates are satisfactorily about 90% and about 80%, respectively. However,  
20 hypertrophic cardiomyopathy is believed to be a cause of sudden death, which poses a clinical problem. Therefore, there is a demand for a radical therapy for the disease. A therapeutic technique of the present invention is also effective to hypertrophic cardiomyopathy and provides an  
25 advantageous therapeutic effect.

The term "dilated phase hypertrophic cardiomyopathy" refers to a type of hypertrophic cardiomyopathy in which the formation of fibrous tissue is  
30 developed in a myocardium in the course of the disease, leading to the thinning of a ventricular wall and a reduction in contractile power, and as a result, a ventricular cavity is enlarged, so that a symptom of dilated cardiomyopathy



appears. Dilated phase hypertrophic cardiomyopathy is said as having a very poor prognosis. There are a number of subclinical cases, posing a clinical problem. Therefore, there is also a demand for a radical therapy for dilated  
5 phase hypertrophic cardiomyopathy. A therapeutic technique of the present invention is effective for dilated phase hypertrophic cardiomyopathy and the like and provides an advantageous therapeutic effect.

10           Conventional therapies and diagnostic techniques for refractory heart failure or the like as described above are described in, for example, "Junkanki Shikkan Saishin no Tiryo [Latest Therapy for Circulatory Diseases] 2002-2003, Shigetake Sasayama and Yoshio Yazaki editors, Nankodo, 2002,  
15 and the like. As described in "Junkanki Shikkan Saishin no Tiryo [Latest Therapy for Circulatory Diseases] 2002-2003, Shigetake Sasayama and Yoshio Yazaki editors, Nankodo, 2002, which was most recently published, there was no radical therapy for refractory heart failure. The present invention  
20 is the first to provide a therapy for the above-described heart diseases, particularly refractory heart failure.

          As used herein, the term "prophylaxis" or "prevention" in relation to a certain disease or disorder  
25 refers to a treatment which keeps such a condition from happening before the condition is caused, or causes the condition to occur at a reduced level or to be delayed.

          As used herein, the term "therapy" in relation to  
30 a certain disease or disorder means that when such a condition occurs, such a disease or disorder is prevented from deteriorating, preferably is retained as it is, more preferably is diminished, and even more preferably

extinguished. As used herein, the term "radical therapy" refers to a therapy which eradicates the root or cause of a pathological process. Therefore, when a radical therapy is made for a disease, there in principle is no recurrence  
5 of the disease.

As used herein, the term "prognosis" is also referred to as "prognostic treatment". The term "prognosis" in relation to a certain disease or disorder refers to a diagnosis  
10 or treatment of such a condition after a therapy.

In a preferred embodiment, the prosthetic tissue of the present invention has a three-dimensional, biological connection. As described in other portions of the  
15 specification, examples of biological connection include, but are not limited to, physical connection via extracellular matrices, electrical connection, and the like. In the present invention, physical connection via extracellular matrices is particularly important in view of the strength  
20 of tissue.

In one embodiment, the prosthetic tissue of the present invention is different from conventional prosthetic tissues in that the former comprises a cell.  
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Preferably, a prosthetic tissue of the present invention consists substantially of a cell or a material derived from the cell. Since the prosthetic tissue is composed substantially of only a cell and a cell-derived  
30 material (e.g., extracellular matrix, etc.), the prosthetic tissue can have an increased level of biocompatibility and affinity. The cell-derived material representatively includes extracellular matrices. Particularly, the

prosthetic tissue preferably comprises a cell and an extracellular matrix at an appropriate ratio thereof. Such an appropriate ratio of a cell and an extracellular matrix is from about 1:9 to about 9:1, preferably about 3:7 to about 7:3, and more preferably about 3:7 to about 5:5. A preferable ratio varies depending on the purpose. Such variations may be prepared by those skilled in the art. An appropriate ratio can be estimated by investigating the ratio of a cell and an extracellular matrix in an organ of interest.

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In another embodiment, the prosthetic tissue of the present invention is preferably isolated. In this case, the term "isolate" means that the prosthetic tissue is separated from a scaffold, a support, and a culture medium used in culture. If a prosthetic tissue of the present invention is substantially free from materials, such as a scaffold and the like, it is possible to suppress adverse reactions after implantation, such as immune rejection reactions, inflammation reactions, and the like.

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In another embodiment, the prosthetic tissue of the present invention is preferably intact. An intact prosthetic tissue can be provided substantially only after the prosthetic tissue production method of the present invention using a three-dimensional promoting agent is provided. Conventionally, when a prosthetic tissue is detached from the culture environment, a physical stimulus is necessarily applied to the tissue, so that the tissue is unavoidably hurt. Therefore, an attempt has been made to compile a plurality of cell sheets prepared by conventional methods. However, such a multilayer of cell sheets cannot be easily used for actual implantation. Therefore, such a problem is overcome by the intactness of the prosthetic tissue

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of the present invention.

In a preferred embodiment, the prosthetic tissue of the present invention has a large size. The term "large size" in relation to a prosthetic tissue typically means that the prosthetic tissue has an area sufficient to cover a site to which the prosthetic tissue is implanted. Such an area is, for example, at least 1 cm<sup>2</sup>, more preferably at least 2 cm<sup>2</sup>, at least 3 cm<sup>2</sup>, at least 4 cm<sup>2</sup>, even more preferably at least 5 cm<sup>2</sup>, and still even more preferably at least 6 cm<sup>2</sup>.

In a preferred embodiment, the prosthetic tissue of the present invention is thick. The term "thick" in relation to a prosthetic tissue typically means that the prosthetic tissue has a thickness which provides a strength sufficient to cover a site to which the prosthetic tissue is implanted. Such a thickness is, for example, at least about 50 μm, more preferably at least about 100 μm, at least about 200 μm, at least about 300 μm, even more preferably at least about 400 μm, still more preferably at least about 500 μm, and still even more preferably about 1 mm.

A prosthetic tissue of the present invention is preferably imperforate. The imperforate prosthetic tissue is suitable for implantation. The imperforate prosthetic tissue is particularly preferable for reinforcement of a defective site of a bag-shaped tissue which needs to be covered.

In another embodiment, the prosthetic tissue of the present invention is flexible. Due to the flexibility, the prosthetic tissue is particularly suitable for reinforcement of motile organs. Examples of motile organs include, but

are not limited to, hearts, blood vessels, muscles, and the like.

In another embodiment, the prosthetic tissue of the present invention has dilation/contraction ability. Due to the dilation/contraction ability, the prosthetic tissue is suitable for organs which expand and contract, including, for example, hearts, muscles, and the like. The dilation/contraction ability cannot be achieved by cell sheet or the like prepared by conventional methods. Preferably, a prosthetic tissue of the present invention has a sufficient strength to withstand the pulsation motion of a heart. The strength sufficient to withstand pulsation motion is, but is not limited to, at least about 50% of the strength of naturally-occurring myocardium, preferably at least about 75%, and more preferably at least about 100%.

In a preferred embodiment, the prosthetic tissue of the present invention has biological connection in all three dimensions. There are some prosthetic tissues prepared by conventional methods, which have biological connection in two dimensions to some degree. However, no tissue having biological connection in all three dimensions can be prepared by conventional methods. Therefore, since the prosthetic tissue of the present invention has biological connection in all three dimensions, the prosthetic tissue is substantially implantable in any application.

Examples of biological connection which is an indicator of a prosthetic tissue of the present invention, include, but are not limited to, interconnection of extracellular matrices, electrical connection, the presence of intracellular signal transduction, and the like. The

interaction of extracellular matrices can be observed with a microscope by staining intracellular adhesion as appropriate. Electrical connection can be observed by measuring electric potential.

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In a preferred embodiment, the prosthetic tissue of the present invention has a sufficient tissue strength for clinical applications. The sufficient tissue strength for clinical applications varies depending on a site to which the prosthetic tissue is applied. Such a strength can be determined by those skilled in the art with reference to the disclosure of the specification and techniques well known in the art. For example, in a preferred embodiment, a strength required by the present invention is at least 80% of the tissue strength of a portion targeted by a clinical application.

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In a specific embodiment, the above-described portion targeted by a clinical application includes the heart, and in some cases, portions other than the heart.

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In another aspect, the present invention provides a cell culture composition for producing prosthetic tissue from a cell. The cell culture composition contains an ingredient (e.g., commercially available medium, etc.) for maintaining or growing the cell, and a three-dimensional promoting agent. The three-dimensional promoting agent has been described in detail in the above description of the prosthetic tissue production method. Therefore, the three-dimensional promoting agent includes ascorbic acid or a derivative thereof (e.g., ascorbic acid 1-phosphate or a salt thereof, ascorbic acid 2-phosphate or a salt thereof, L-ascorbic acid or a salt thereof, etc.). The culture

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composition of the present invention contains ascorbic acid 2-phosphate or a salt thereof at a concentration of at least 0.1 mM. Alternatively, in the case of a condensed culture composition, the condensed culture composition contains  
5 ascorbic acid 2-phosphate or a salt thereof at a concentration which becomes at least 0.1 mM after preparation. Alternatively, the lower limit concentration of ascorbic acid 2-phosphate or a salt thereof may be 0.01 mM, 0.05 mM, 0.2 mM, or 0.3 mM, even more preferably 0.5 mM, or  
10 alternatively 1.0 mM.

In a preferred embodiment, the three-dimensional promoting agent contained in the cell culture composition of the present invention includes ascorbic acid 1-phosphate  
15 or a salt thereof and ascorbic acid 2-phosphate or a salt thereof.

Ascorbic acid 2-phosphate used in the present invention is used concomitantly with ascorbic acid  
20 1-phosphate. In this case, ascorbic acid 1-phosphate and ascorbic acid 2-phosphate may be preferably present at a specific ratio. Such a preferable ratio is, for example, in the range of from 1:10 to 10:1. Alternatively, the preferable ratio is such that the molar amount of ascorbic  
25 acid 1-phosphate is smaller than the molar amount of ascorbic acid 2-phosphate.

In another embodiment, the three-dimensional promoting agent used in the present invention includes  
30 ascorbic acid 2-phosphate. However, there was no report that ascorbic acid 2-phosphate is explicitly used for formation of a tissue. In the present invention, by adding a certain amount of ascorbic acid 2-phosphate, it is possible to prevent

an extracellular matrix from being produced in an excessively large amount, the composition has an implantable level of extracellular matrices, and therefore, the ratio of the cell to the extracellular matrix results in an implantable level of strength and the like. These effects are unexpected.

In a preferred embodiment, ascorbic acid 2-phosphate used in the present invention is typically present at a concentration of at least 0.01 mM, preferably at least 0.05 mM, more preferably at least 0.1 mM, even more preferably at least 0.2 mM, and still more preferably at least 0.5 mM, and still even more preferably 1.0 mM.

In a certain preferred embodiment, the three-dimensional promoting agent of the present invention includes ascorbic acid 1-phosphate or a salt thereof, ascorbic acid 2-phosphate or a salt thereof, and L-ascorbic acid or a salt thereof.

(Prosthetic tissue for "wrapping")

In another aspect, the present invention provides a prosthetic tissue for reinforcement of a portion of an animal organism. The prosthetic tissue capable of such reinforcement is a technique achieved only after the prosthetic tissue production method of the present invention is provided.

In a preferred embodiment, the above-described portion includes bag-shaped organs. For bag-shaped organs, it is important for the prosthetic tissue to possess intactness and/or nonporousness. A prosthetic tissue having such a property and a certain size cannot be provided by conventional techniques. Therefore, it can be said that



a substantial therapy for bag-shaped organs can be achieved only after the above-described prosthetic tissue of the present invention is provided. Therefore, for bag-shaped organs which have specific diseases (e.g., refractory diseases (e.g., dilated cardiomyopathy, etc.), etc.), a  
5 therapy can be first provided other than organ transplantation.

In a specific embodiment, examples of the  
10 above-described bag-shaped organ include, but are not limited to, hearts, livers, kidneys, and the like.

In a specific embodiment of the present invention, the above-described reinforcement may be achieved by  
15 disposing a prosthetic tissue of the present invention to cover the above-described portion. It is not possible to use a prosthetic tissue provided by conventional methods to perform treatment by covering the above-described portion (i.e., "wrapping" application). Thus, the prosthetic  
20 tissue of the present invention can provide applications which cannot be achieved by conventional techniques.

Therefore, in the above-described specific embodiment, the prosthetic tissue of the present invention  
25 is resistant to dilation/contraction of the above-described portion.

In a preferred embodiment, the prosthetic tissue of the present invention advantageously has biological  
30 connection.

In another preferred embodiment, the biological connection includes at least one of interconnection of

extracellular matrices, electrical connection, and intracellular signal transduction.

5 In another preferred embodiment, the prosthetic tissue for reinforcement of the present invention is formed by culturing a cell in the presence of a three-dimensional promoting agent.

10 In another embodiment, the prosthetic tissue for reinforcement of the present invention comprises a cell (autologous cell) derived from an animal to be treated (e.g., a human). More preferably, a prosthetic tissue for reinforcement of the present invention comprises only a cell(s) (autologous cell) derived from an animal to be treated  
15 (e.g., a human).

("Wrapping" therapy)

In another aspect, the present invention provides a method for reinforcement of a portion of an animal organism.  
20 The method comprises the steps of: A) disposing a prosthetic tissue to cover the portion; and B) holding the prosthetic tissue for a time sufficient to connect to the portion. The step of disposing the prosthetic tissue to cover the portion can be carried out using a technique well known in the art.  
25 The sufficient time varies depending on a combination of the portion and the prosthetic tissue, and can be easily determined as appropriate by those skilled in the art depending on the combination. Examples of such a time include, but are not limited to, 1 week, 2 weeks, 1 month, 2 months,  
30 3 months, 6 months, 1 year, and the like. In the present invention, a prosthetic tissue preferably comprises substantially only cell(s) and material(s) derived from the cell. Therefore, there is no particular material which needs

to be extracted after operation. The lower limit of the sufficient time is not particularly important. In this case, it can be said that the longer the time, the more preferable the prosthetic tissue. If the time is sufficiently extremely long, it can be said that reinforcement is substantially completed. Therefore, the time is not particularly limited.

In another embodiment, in a reinforcement method of the present invention, the above-described portion preferably includes bag-shaped organs (e.g., hearts, livers, kidneys, etc.). In order to reinforce such a bag-shaped tissue, it is necessary to wrap the organ (e.g., cover an injured portion). A prosthetic tissue resistant to wrapping applications is first provided by the present invention. Therefore, the reinforcement method of the present invention is advantageous over conventional techniques.

Particularly, in the reinforcement method of the present invention, a prosthetic tissue of the present invention is resistant to dilation/contraction of the above-described portion. Examples of such dilation/contraction include, but are not limited to, the pulsation motion of a heart, the contraction of a muscle, and the like.

In another preferred embodiment, in the reinforcement method of the present invention, a prosthetic tissue of the present invention has biological connection (e.g., interconnection of extracellular matrices, electrical connection, intracellular signal transduction, etc.). The biological connection is preferably provided in all three dimensions.

In another preferred embodiment, the reinforcement method of the present invention further comprises culturing a cell in the presence of a three-dimensional promoting agent to form a prosthetic tissue of the present invention. An  
5 implantation/regeneration technique using the method which comprises the step of culturing a cell in the presence of a three-dimensional promoting agent cannot be provided by conventional techniques. The method provides a therapy for diseases (e.g., refractory heart diseases (e.g., dilated  
10 cardiomyopathy, etc.), etc.), which cannot be achieved by conventional therapies.

In a preferred embodiment, in the reinforcement method of the present invention, the cell used in the  
15 prosthetic tissue of the present invention is derived from an animal to which the prosthetic tissue is to be implanted (i.e., an autologous cell). By using an autologous cell, adverse side effects, such as immune rejection reactions or the like, can be avoided.

20 In another preferred embodiment, the portion is a heart. The heart has a disease or disorder, such as heart failure, ischemic heart disease, myocardial infarct, cardiomyopathy, myocarditis, hypertrophic cardiomyopathy,  
25 dilated phase hypertrophic cardiomyopathy, dilated cardiomyopathy, or the like.

For some organs, it is said that it is difficult to radically treat a specific disease, disorder, or condition  
30 thereof (e.g., refractory heart diseases). However, the present invention provides the above-described effect, thereby making possible a treatment which cannot be achieved by conventional techniques. It has been clarified that the

present invention can be applied to radical therapy. Therefore, the present invention has usefulness which cannot be achieved by conventional medicaments.

5 (Combined therapy)

In another aspect, the present invention provides a regeneration therapy which uses a cytokine, such as HGF or the like, in combination with a prosthetic tissue.

10 Cytokines used in the present invention are already commercially available (e.g., HGF-101 from Toyo Boseki, etc.). However, these cytokines can be prepared by various methods and can be used in the present invention if they are purified to an extent which allows them to be used as a medicament.

15 HGF can be obtained as follows: primary cultured cells or an established cell line capable of producing HGF is cultured; and HGF is separated from the culture supernatant or the like, followed by purification. Alternatively, a gene encoding HGF is incorporated into an appropriate vector by

20 a genetic engineering technique; the vector is inserted into an appropriate host to transform the host; recombinant HGF of interest can be obtained from the supernatant of the transformed host culture (e.g., Nature, 342, 440(1989); Japanese Laid-Open Publication No. 5-111383;

25 Biochem-Biophys. Res. Commun., 163, 967 (1989), etc.). The above-described host cell is not particularly limited and can be various host cells conventionally used in genetic engineering techniques, including, for example, *Escherichia coli*, yeast, animal cells, and the like. The thus-obtained

30 HGF may have one or more amino acid substitutions, deletions and/or additions in the amino acid sequence as long as it has substantially the same action as that of naturally-occurring HGF. Examples of a method for

introducing HGF into patients in the present invention include, but are not limited to, a Sendai virus (HVJ) liposome method with high safety and efficiency (Molecular Medicine, 30, 1440-1448(1993); Jikken Igaku (Experimental Medicine), 5 12, 1822-1826(1994)), an electrical gene introduction method, a shotgun gene introduction method, and the like. Preferably, the HVJ liposome method is used.

Hereinafter, the present invention will be described  
10 by way of examples. Examples described below are provided only for illustrative purposes. Accordingly, the scope of the present invention is not limited except as by the appended claims.

#### 15 EXAMPLES

In the examples below, animals were treated in accordance with rules defined by Osaka University (Japan) and were cared for in the spirit of animal protection.

20 (Example 1: Production and utilization of prosthetic tissue and three-dimensional structure made of cardiomyocyte sheet - tissue engineered contractile cardiomyocyte sheet regenerates impaired myocardium -)

25 (Bioengineered contractile cardiomyocyte sheet regenerates infarcted myocardium)

In Example 1, the present inventors investigated  
30 (1) whether or not a cardiomyocyte sheet (prosthetic tissue) survives after implantation and shows histological electrical connection with impaired myocardium; (2) whether or not the implanted cardiomyocyte sheet (prosthetic tissue) can induce an improvement in a cardiac function. As a result,

it was demonstrated that the present invention provides the electrical connection and the improvement of cardiac function.

5           The present inventors introduced the concept of  
bioengineered tissue implantation into the treatment of  
impaired myocardium. It was demonstrated that a tissue  
engineered contractile cardiomyocyte sheet without a  
scaffold provides histological electrical connection with  
10   impaired myocardium and regeneration of an infarcted  
myocardium.

(Materials and methods)

(Myocardial infarct model)

15           30 male Lewis rats (300 g, 8 weeks old; Seac Yoshitomi  
Ltd, Fukuoka, Japan) were used in this study. Humane animal  
care complied with "Principles of Laboratory Animal Care"  
prepared by the National Society for Medical Research and  
"Guide for the Care and Use of Laboratory Animals" (NIH  
20   Publication, No. 86-23, 1985, revised) prepared by the  
Institute of Laboratory Animal Resource and published by  
the National Institute of Health. Acute myocardial infarct  
was induced as described in Weisman H.F., Bush D.E., Mannisi  
J.A., et al., Cellular Mechanism of Myocardial Infarct  
25   Expansion, Circulation, 1988; 78: 186-201. Briefly, the  
rats were anesthetized with sodium pentobarbital, followed  
by positive pressure breathing through an endotracheal tube.  
A left 4th intercostal space thoracotomy was used and the  
left anterior descending coronary artery (LAD) was  
30   completely ligated with an 8-0 polypropylene thread at a  
distance of 3 mm from the root of the LAD.

(Preparation of rectangle-designed PIPAAm grafted

polystyrene cell culture dishes)

A rectangle-designed PIPAAm grafted cell culture dish was prepared with a specific procedure as described in, for example, Okano T., Yamada N., Sakai H., Sakurai Y., J. Biomed. Mater. Res., 1993; 27:1243-1251. Briefly, an  
5 IPAAm monomer solution in 2-propanol (kindly provided by Kohjin, Tokyo, Japan) was spread onto tissue culture polystyrene (TCPS) dishes (Falcon 3002, Becton Dickinson). Thereafter, these dishes were subject to irradiation  
10 (electron beam dose: 0.25 MGy) using an Area Beam Electron Processing System (Nisshin High Voltage), resulting in polymerization of IPAAm and covalent bonding of IPAAm to the dish surface. The PIPAAm grafted dishes were rinsed with cold distilled water to remove non-grafting IPAAm, followed  
15 by drying the dishes in nitrogen gas. In the second step, the PIPAAm grafted surface was masked with a rectangular coverglass (24×24 mm, Matsunami, Tokyo, Japan). Acrylamide (AAm) monomer solution of 2-propanol was spread onto the surface of the masked dish. Thereafter, the dish surface  
20 was electron beam irradiated and washed. As a result, the rectangular region at the center of each dish was PIPAAm-grafted (temperature responsive), while the surrounding border was poly-AAm grafted (non-cell adhesive). The rectangle-designed PIPAAm grafted dish was sterilized  
25 with ethylene oxide gas before use in culture.

(Primary culture of newborn rat ventricular muscle cells)

Primary newborn rat cardiomyocytes were prepared in  
30 accordance with a procedure previously described in, for example, Shimizu T., Yamato M., Akutsu T. et al., Circ. Res., Feb 22, 2002; 90(3):e40. Briefly, 1 to 2 day old newborn rats were sacrificed while deeply anesthetized, and the



hearts were rapidly removed, followed by digestion with Hanks solution containing collagenase (class II, Worthington Biochemical) at 37°C. The isolated cells were suspended in culture medium containing 6% FCS, 40% Medium 199 (Gibco BRL),  
5 0.2% penicillin-streptomycin solution, 2.7 mmol/L glucose, and 54% balanced salt solution (116 mmol/L NaCl, 1.0 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 0.8 mmol/L MgSO<sub>4</sub>, 1.18 mmol/L KCl, 0.87 mmol/L CaCl<sub>2</sub>, and 26.2 mmol/L NaHCO<sub>3</sub>). The cell suspension was plated at a density of 8×10<sup>6</sup> cells/dish, followed by incubation in a  
10 humidified atmosphere containing 5%CO<sub>2</sub> at 37°C.

(Primary culture of fibroblasts)

Primary fibroblasts were prepared in accordance with a procedure previously published in, for example,  
15 Yablonka-Reuveni Z., Nameroff M., Skeletal Muscle Cell Populations Separation and Partial Characterization of Fibroblast-Like Cells from Embryonic Tissue Using Density Centrifugation, Histochemistry, 1987; 87:27-38. Briefly, a suspension of cells derived from leg muscle of 8 week old  
20 Lewis rats was separated into fibroblasts and muscle cells by Percoll<sup>TM</sup> density centrifugation (Amersham Biosciences Sweden). The isolated fibroblasts were used for a control fibroblast sheet.

25 (Production of cardiomyocyte sheet)

The isolated cardiomyocytes of the newborn rat were cultured on the rectangle-designed PIPAAm grafted polystyrene cell culture dish. By lowering the temperature to 20°C, the cells were detached as a rectangular cell sheet.  
30 Two cardiomyocyte sheets were piled up to produce a thicker heart graft. Cross sectional observation of the double-layered cardiac sheet demonstrated intimate connection and homogenous heart-like tissue. Synchronous

motion of a four-layer cardiomyocyte sheet was detected by the naked eye (data not shown). In the case of the fibroblast sheet, isolated fibroblasts were cultured on the same dish for 2 days, and were detached as a rectangular cell sheet  
5 by the same method.

(Implantation of cardiomyocyte sheet)

The cardiomyocyte sheet was implanted into Lewis rats 2 weeks after LAD ligation. Specifically, a left 4th  
10 intercostal space thoracotomy was performed on the rat under general anesthetization. The infarct region was visually identified based on the surface scar and the abnormal wall motion. The cardiomyocyte sheet or the fibroblast sheet was implanted into an infarcted myocardium. A control group was  
15 not treated.

The cardiac function was evaluated 2 weeks, 4 weeks, and 8 week after implantation. 8 weeks after implantation, the heart was collected and sectioned, and processed for  
20 histological and immunohistological tests.

In order to identify the implanted cardiomyocyte sheet, EGFP newborn rat cardiomyocytes were isolated with the same protocol, and a cardiomyocyte sheet was prepared.  
25 The present inventors implanted the EGFP newborn rat cardiomyocyte sheet into an infarcted myocardium of nude rats. The present inventors could detect EGFP positive cardiomyocytes in the infarcted myocardium (Figure 6, lower left portion and lower right portion).

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(Measurement of cardiac function of rat heart)

Rats were anesthetized with sodium pentobarbital. Anesthetization was supplemented with ethanol to maintain

light anesthetization. The rats were lightly secured in a supine position and the precordia of the rats were shaved. Heart ultrasonography was performed using a commercially available echocardiograph SONOS 5500 (PHILIPS Medical Systems, USA). A 12-MHz annular array transducer was placed on an acoustic coupler gel layer applied onto the left hemithorax. The transducer was carefully made to have adequate contact with the thorax while avoiding an excessive level of pressure on the thorax. The rats were imaged in a shallow left lateral decubitus position. The heart was first imaged in a two-dimensional mode in a short axis cross section at a level of the greatest left ventricle (LV) diameter. The systolic left ventricle (LV) area and the diastolic left ventricle (LV) area were determined at the same time. The volume of the left ventricle (LV) was estimated based on a short axis area of the left ventricle (LV) (Gorcsan J. 3rd, Morita S., Mandarino W.A., Deneault L.G., Kawai A., Kormos R.L., Griffith B.P., Pinsky M.R., Two-dimensional Echocardiographic Automated Border Detection Accurately Reflects Changes in Left ventricular Volume, J. Am. Soc. Echocardiogr., 1993; 6: 482-9). The resultant image was used to locate an Mmode cursor perpendicular to the left ventricle (LV) anterior wall and the left ventricle (LV) posterior wall. All measurements are performed online using a monitor. Diastolic measurements were performed at the time of an apparent maximum left ventricle (LV) diastolic dimension. A left ventricle (LV) endsystolic dimension was measured at the time of the most anterior systolic excursion of the left ventricle (LV) posterior wall. The left ventricle (LV) dimension in diastole (LVDD) and the left ventricle (LV) dimension in systole were measured. Dimension data and area data were represented by an average of measured values of two or three selected beats. A left ventricle (LV) ejection

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fraction (EF) was calculated by:

$$\text{LVEF(\%)} = [(\text{LVDD}^3 - \text{LVDS}^3)/\text{LVDD}^3] \times 100.$$

5           LV% fractional shortening (FS) was calculated by:

$$\text{LV\%FS} = [(\text{LVDD} - \text{LVDS})/\text{LVDD}] \times 100.$$

10           (Endocardiography quantification of regional left ventricular wall motion using color kinesis)

          Color kinesis is an extension of this technology, which compares tissue backscatter values between successive acoustic frames as a means of automatically tracking and displaying endocardial motion in real time. Color kinesis  
15           was incorporated into a commercially available ultrasound system (SONOS 5500, PHILIPS Medical Systems, USA) (Auchincloss 1988, Transplantation 46:1; Robert M.L., Philippe V., Lynn W., James B., Claudia K., Joanne S., Rick K., David P., Victor M.A., et al., "Echocardiographic  
20           quantification of regional left ventricular wall motion with color kinesis", Circulation, 1996, 93:1877-1885).

          In all study subjects, ultrasound imaging was performed with a 12-MHz annular array transducer.  
25           Midpapillary parasternal short-axis was obtained during end expiration in the lateral decubitus position. After image quality was optimized, the acoustic quantification system for endocardial boundary detection was activated. Gain control (total and lateral gain, time gain compensation)  
30           were adjusted to optimize tracking of the blood-endocardial interface within a predefined region of interest. Color kinesis then was activated for on-line color encoding of endocardial excursion throughout systole. Image sequences

containing color kinesis data were obtained throughout the cardiac cycle and stored in a digital format on optical disks for off-line analysis.

5 (Histopathology)

Left ventricular myocardium specimens were obtained at 2 and 8 weeks after cardiomyocyte sheet implantation. Each specimen was placed in 10% neutral formaldehyde and embedded in paraffin. A few serial sections were cut from  
10 each specimen and stained with hematoxylin and eosin for light microscopic examination.

To label vascular endothelial cells, immunohistochemical staining of factor VIII-related antigen  
15 was performed. Frozen sections were fixed with a 2% paraformaldehyde solution in PBS for 5 minutes at room temperature, immersed in methanol with 3% hydrogen peroxide for 15 minutes, then washed with PBS. The samples were covered with bovine serum albumin solution (DAKO LSAB Kit  
20 DAKO CORPORATION, Denmark) for 10 minutes to block nonspecific reactions. The specimens were incubated overnight with an EPOS-conjugated antibody against factor VIII-related antigen coupled with HRP (DAKO EPOS Anti-Human Von Willebrand Factor/HRP, DAKO Denmark). After the samples  
25 were washed with PBS, they were immersed in diaminobenzidine solution (0.3 mg/ml diaminobenzidine in PBS) to obtain positive staining.

To detect Connexin 43, immunohistochemical staining  
30 of Connexin 43-related antigen was performed. Frozen sections were immersed in methanol with 3% hydrogen peroxide for 5 minutes, and then washed with PBS. The specimens were incubated for 20 minutes with a mouse monoclonal antibody

to Connexin 43 (CHEMICON International, Inc. USA). After the samples were washed with PBS, they were immersed in biotinylated anti-mouse immunoglobulins (DAKO Denmark) for 10 minutes, and then washed with PBS. The samples were  
5 immersed in peroxidase-conjugated streptavidin (DAKO Denmark) for 10 minutes. After the samples were washed with PBS, they were immersed in diaminobenzidine solution (0.3 mg/ml diaminobenzidine in PBS) to obtain positive staining.

10 (Electrophysiological analysis)

One microelectrode (100  $\mu$ m in diameter, Unique Medical Co., Ltd., Tokyo) for capture of the electrical potentials was positioned over the cardiomyocyte sheets implanted scar, fibroblast sheets implanted scar, or no  
15 treatment scar. The other two electrodes were put on left subcostal and right femoral region. For stimulation of the host myocardium, two microelectrodes were positioned over an atrium. For detection of host electrocardiogram, three electrodes were attached on right upper breast, left  
20 subcostal, and right femoral regions. Both electrograms were amplified by bioelectric amplifiers (UA-102, Unique Medical Co., Ltd., Tokyo) and were recorded by a data acquisition system (UAS-108S, Unique Medical Co., Ltd., Tokyo). The atrium was stimulated at the rate of 300 bpm  
25 by stimulator (NIHON KODEN Japan). Thereafter, the electrical potential was captured in the regions of interest.

Thereafter, in order to analyze the threshold, two microelectrodes for stimulation were positioned over the  
30 no treatment scar, cardiomyocyte sheet implanted scar, or fibroblast sheets implanted scar. For detection of paced host electrocardiogram, one electrode was attached on a normal myocardium, and the other two electrodes were put

on left subcostal and right femoral regions. Thereafter, the present inventors stimulated the no treatment scar, cardiomyocyte sheet implanted scar, or fibroblast sheets implanted scar at the rate of 300 bpm by the same stimulator.

5

(Data analysis)

Data are expressed as means  $\pm$  standard deviation (SD). In order to assess the significance of the differences between individual groups, statistical evaluation was performed with the nonparametric Mann-Whitney two-sample test. Statistical significance was determined as a p-value less than 0.05.

10

(Results)

15

(Characteristics of Cardiac grafts)

Detached cardiomyocyte sheets shrank from 5.76 cm<sup>2</sup> to 1.11 $\pm$ 0.05 cm<sup>2</sup> (n=3) in area due to cytoskeletal reorganization. On the other hand, the thickness increased from 20.1 $\pm$ 0.9  $\mu$ m to 52.4 $\pm$ 6.0  $\mu$ m (n=3). The cardiac sheets contracted spontaneously by macroscopic observation.

20

(Histological assessment)

The present inventors could detach a mono-layered cardiomyocyte sheet under low temperature (Figure 6, upper left portion). The cardiomyocyte sheet was attached on an infarcted myocardium immediately after implantation without ligature (Figure 6, upper right portion). 2 weeks after implantation, histological examination of Hematoxylin-Eosin (HE) stain of the implanted cardiomyocyte sheet showed good attachment in alignment on the infarcted myocardium without accumulation of inflammatory cells. The yellow arrow indicates a collagen sheet, which is necessary to deliver the cardiomyocyte sheet to the infarcted

25

30

myocardium (Figure 7). Visual inspection of the implanted cardiomyocyte sheet at eight weeks after implantation revealed the intimate attachment between the implanted cardiomyocyte sheet and a recipient heart. HE stain of the  
5 implanted cardiomyocyte sheet after eight weeks of implantation demonstrated the integration of the cardiomyocyte sheet with the host myocardium in alignment in the center of the scar (Figure 7, lower right portion). Immunohistochemical staining showed randomly oriented  
10 Connexin 43 in the implanted cardiomyocyte sheet and around a contact region between the implanted cardiomyocyte sheet and the recipient heart (Figure 7, lower middle portion). Factor VIII immunohistochemical staining demonstrated many Factor VIII positive cells in and around the implanted  
15 cardiomyocyte sheet (Figure 7, lower left portion).

(Functional recovery of infarcted myocardium)

B-mode analysis showed that dilatation of the left ventricle was well suppressed and global wall motion was  
20 well preserved in the T group compared with the C group (Figure 8). An ejection fraction (EF), a fractional shortening (FS), and a left ventricular endsystolic area (LVESA) at a baseline were not significantly different between the three groups.

25

2 and 4 weeks after implantation, 2D endocardiography showed a significant improvement of EF and FS in the T group compared with those in the other groups. LVESA was significantly smaller in the T group than that in the other  
30 groups. These functional improvements were preserved 8 weeks after implantation (Figure 9).

(Electrophysiological experiments)



Electrophysiological experiments showed the one peak component of QRS wave of the treated scar area in the T group in spite of two peaks of QRS wave like branch blocks in the C group (Figure 11). An effect was observed in the F group, though the level of the effect was lower than that of the T group. Furthermore, electrophysiological experiments demonstrated an improvement of the amplitude (volts) of the QRS complex in the T group in spite of low amplitude in the F and C group (C group vs T group:  $2.79 \pm 0.9$  V vs  $0.83 \pm 0.64$  V,  $P < 0.05$ ) (Figure 11).

In the T group, when an atrium was stimulated at the frequency of 300 bpm by a stimulator, synchronous electrical spikes were detected in the implanted area (Figure 10, lower left portion). Furthermore, a threshold for pacing of a recipient heart was lower in the T group than the F and C groups (C group vs F group vs T group:  $4.9 \pm 0.9$  V vs  $3.0 \pm 0.7$  V vs  $1.7 \pm 0.5$  V,  $P < 0.05$ ) (Figure 10, right portion).

#### (Results)

A temperature-responsive domain made of poly(N-isopropylacrylamide) was grafted on polystyrene cell culture surfaces. Newborn rat cardiomyocytes were cultured on these dishes and detached as a rectangular cell sheet below  $20^{\circ}\text{C}$  without trypsin. Two sheets were piled up to make thicker cardiac grafts. These cardiomyocyte sheets contracted spontaneously. Cross sectional observation of the two sheets demonstrated intimate connection and homogenous heart-like tissue.

30

Two weeks after left anterior descending (LAD) ligation, two different treatments were conducted: 1) cardiomyocyte sheet implantation (T group,  $n=10$ ); and 2)

fibroblast sheet implantation (F group, n=10). The control group was not treated (C group, n =10). Endocardiography demonstrated that cardiac function was significantly ameliorated in the T group two, four, and 8 weeks after implantation. Color kinesis showed that regional systolic wall motion was excellently recovered as compared with values before implantation. Cardiomyocyte sheets attached on the infarcted myocardium were in alignment and seemed to be homogeneous tissue in the myocardium. Immunohistochemical staining demonstrated neoangiogenesis and randomly oriented Connexin 43 in the implanted cardiomyocyte sheets. Electrophysiological experiments showed the improvement of the R-wave and the one peak component of the QRS complex in the treated scar area in the T group in spite of the two peak components of the QRS complex like branch block in the F and C group. Furthermore, a threshold for pacing of a recipient heart was lower in the T group than the F and C group (C group vs F group vs T group:  $4.9 \pm 0.9$  V vs  $3.0 \pm 0.7$  V vs  $1.7 \pm 0.5$  V,  $P < 0.05$ ).

#### (Discussion)

The recent development of cellular cardiomyopathy introduced by needle injection has provided a novel approach to restore impaired cardiac function (Taylor D.A., Atkins B.Z., Hungspreugs P., Jones T.R., Reedy M.C., Hutcheson K.A., Glower D.D., Kraus W.E., Regeneration of Functional Myocardium: Improved Performance after Skeletal Myoblast Transplantation, *Nature Med.*, 1998; 4:929-933). Additional potential advantages of the cardiomyocyte sheet implantation method are better control of the tissue formation process (the shape, size, and consistency of a graft), an easy implantation technique, and implantation of a number of cells with minimum cell loss. In contrast, an injection method

provides a loss of a certain amount of cells or its surface protein (e.g., Connexin 43) by means of trypsin treatment. In addition to myocardial infarction, the cardiomyocyte sheet implantation method may be useful for repair of global myocardial dysfunction (e.g., dilated cardiomyopathy). To achieve clinical applications, total implanted myocardial tissue mass is critically important for the repair of impaired myocardium, and enhancement of angiogenesis may make it possible for cardiac sheets to be thicker and deliver more cells to impaired myocardium.

In this example, the present inventors have developed a contractile cardiomyocyte sheet without a scaffold and have analyzed cardiac function and histological assessment after implantation. The cardiomyocyte sheet has attached to an infarcted myocardium accompanied with angiogenesis. The cardiomyocyte sheet looked like homogeneous myocyte tissue expressing Connexin 43 and contracted synchronously *in vivo*. Cardiomyocytes in the cardiomyocyte sheet exhibited alignment and few inflammatory cells accumulated in the implanted cardiomyocyte sheet. The cardiomyocyte sheet implantation promised excellent improvement of systolic and diastolic cardiac performance. Electrophysiological experiments revealed that the cardiomyocyte sheet improves electrical conductivity in scar and reconstructs one peak component of the QRS complex in the treated scar area. These data proved the present inventors' hypothesis that contractile cardiomyocyte sheets exhibit histological and electrical integration with impaired myocardium accompanied with angiogenesis and expression of Connexin 43 and induces the significant improvement of cardiac performance. To the present inventors' best knowledge, this is the first report in which

myocardial regeneration therapy was successful in impaired myocardium using a tissue engineered cardiac sheet.

5       The main factors of integration in the regenerative therapy are dynamic integration, electrical integration, and histological integration.

10       Concerning dynamic integration, cardiomyocytes of the implanted cardiomyocyte sheet showed alignment and promoted significant improvement of regional and global systolic function in infarcted myocardium. Impaired remodeling is responsible for the cardiac structural deformation and cardiac function deterioration in an infarcted heart (Tyagi S.C., Extracellular Matrix Dynamics in Heart Failure: A Prospect for Gene Therapy, J. Cell. Biochem., 1998, 68:403-410). Kelley et al. showed that restraint of the dilation of a left ventricle (LV) with mesh placed over an infarcted myocardium preserves the left ventricle (LV) geometry and resting function in a sheep myocardial infarction model (Kelley S.T., Malekan R., Gorman J.H. et al., Restraining Infarct Expansion Preserves Left Ventricular Geometry and Function after Acute Anteroapical Infarction, Circulation, 1999, 99:135-142). Although both the preservation of the left ventricle (LV) geometry and the improvement of a regional systolic function in 25       dysfunctioned tissues may be essential for repair of systolic performance in an impaired heart, only the attenuation of left ventricular (LV) dilatation is not adequate treatment. The present inventors' data, in which non-contractile sheets failed to improve the systolic performance compared with 30       contractile sheets, supports this finding. Moreover, the present inventors' data demonstrated significant angiogenesis in infarcted myocardium. One of the mechanisms

for the improvement of cardiac function may be angiogenesis induced by an implanted cardiomyocyte sheet. To sum up, the significant improvement of systolic function induced by an implanted cardiomyocyte sheet is responsible for the preservation of left ventricular (LV) geometry, the improvement of regional systolic function, and induction of angiogenesis.

Concerning electrical integration, the present inventors' study demonstrated that an electrical conductor (i.e., a cardiomyocyte sheet expressing Connexin 43) promoted conductivity in scar, leading to the improvement of the amplitude of the QRS complex and the repair of two peak components of the QRS complex like branch block in infarcted myocardium. Branch block patterns are likely to be related to fibrosis or necrosis in myocardium (Agarwal A.K., Venugopalan P., Right Bundle Branch Block: Varying Electrocardiogram Patterns., An Etiological Correlation, Mechanisms and Electrophysiology, International Journal of Cardiology, 1999, 71:33-39). The histological change was reflected to the amplitude of the QRS complex in electrocardiogram recording (Sakamoto A., Ono K., Abe M., Jasmin G., Eki T., Murakami Y., Masaki T., Toyo-oka T., Hanaoka F., Both Hypertrophic and Dilated Cardiomyopathies Are Caused by Mutation of the Same Gene, delta-sarcoglycan, in Hamster: An Animal Model of Disrupted Dystrophin-associated Glycoprotein Complex, Proc. Natl. Acad. Sci. USA, Dec 9, 1997, 94(25):13873-8). The facts of synchronous wall motion demonstrated in the US, repair of two peak components of the QRS complex like branch block, and decrease of a threshold in scar might reveal electrical connection between an implanted cardiomyocyte sheet and a host myocardium. For this reason, cell sheets kept good surface condition with

Connexin 43 when the present inventors used temperature-responsive dishes, and not trypsin, to detach the cell sheets from culture dishes.

5                   Concerning histological integration, implanted  
cardiomyocyte sheets showed good attachment with infarcted  
myocardium with angiogenesis. Already, the present  
inventors demonstrated that histological integration by the  
enhancement of cell/cell and cell/ECM interaction by a growth  
10                   factor in blood is essential for myocardial regeneration  
of infarcted myocardium (Miyagawa S., Sawa Y., Taketani S.,  
Kawaguchi N., Nakamura T., Matsuura N., Matsuda H.,  
Myocardial Regeneration Therapy for Heart Failure,  
Hepatocyte Growth Factor Enhances the Effect of Cellular  
15                   Cardiomyoplasty, Circulation, 2002, 105: 2556-2561).  
Kushida et al. reported that adhesive agents in cell sheets  
were well preserved after detachment from  
temperature-responsive dishes (Kushida A., Yamato M., Konno  
C., Kikuchi A., Sakurai Y., Okano T., J. Biomed. Mater. Res.  
20                   45:355-362, 1999). Therefore, these sheets show good  
attachment and integration with several organs with good  
preservation of adhesive molecules in its surface (Shimizu  
T., Yamato M., Kikuchi A., et al., Two-dimensional  
Manipulation of Cardiac Myocyte Sheets Utilizing  
25                   Temperature-responsive Culture Dishes Augments the  
Pulsatile Amplitude, Tissue Eng., 2001, 7(2):141-51, 24;  
and von Recum H.A., Kim S.W., Kikuchi A., Okuhara M., Sakurai  
Y., Okano T., Retinal Pigmented Epithelium Culture on  
Thermally Responsive Polymer Porous Substrates, J. Biomater.  
30                   Sci. Polym. Ed 9: 1998, 1241-1254). Moreover, delivery of  
cells with preservation of cellular community may be  
important for cell survival and viability in contrast to  
cell delivery through an injection method. These results

indicate that cardiomyocyte sheets without scaffolds make a syncytium with a host myocardium.

5 A cell source for sheets is a very important matter in clinical applications. Recently, myoblasts are most widely used for clinical applications of cell transplantation. Myoblasts have a potential of ischemic tolerance rather than cardiomyocytes. Therefore, myoblasts are one of the suitable cell sources for cell sheet implantation in clinical  
10 applications at present.

In conclusion, the present inventors have demonstrated that contractile cardiomyocyte sheets integrated with impaired myocardium dynamically,  
15 electrically, and histologically.

Cardiomyocyte sheet implantation may be a novel and promising strategy for repairing functional performance and cardiac structure in impaired myocardium.  
20

Fibroblast sheets also provide a slight improvement which is less than that of heart cell sheets. Therefore, it was demonstrated that fibroblast sheets have a potential to be utilized at least for first aid.  
25

#### (Conclusion)

A prosthetic tissue using newborn rat cardiomyocytes integrated with impaired myocardium and ameliorated cardiac function in an ischemic cardium model. Although the present  
30 inventors herein used cardiomyocytes, a prosthetic tissue organized using a cell sheet technique introduces a novel concept of tissue implantation and is useful in the field of regenerative medicine. This technique is schematically

shown in Figures 1A and 1B.

(Example 2)

5 (Self-derived myoblast sheet regenerates impaired  
myocardium: A way to clinical application - Demonstrating  
examples using rats)

Recent progress in tissue engineering is likely to  
provide implantable functional tissue comprising various  
cells other than myocardial cells, and extracellular matrices.  
10 The present inventors designed autologous myoblast sheets.  
The present inventors considered that these sheets are  
beneficial for clinical applications. In this example,  
therefore, myoblasts were used as material to construct a  
prosthetic tissue or three-dimensional structure and an  
15 effect of the prosthetic tissue or three-dimensional  
structure on clinical applications was demonstrated.

(Methods)

An impaired heart was created in 28 rats by ligating  
20 the left anterior descending (LAD) for 2 weeks. A temperature  
responsive domain made of a polymer (N-isopropylacrylamide)  
was coated on culture dishes. Skeletal myoblasts (SMs)  
isolated from leg muscle were cultured and detached from  
the dishes as a single monolayer cell-sheet (tissue) at 20°C  
25 (Figure 13). Two myoblast sheets were implanted in nine rats  
(myoblast sheet (S) group =  $10^7$  cells). Myoblast cells were  
injected into 9 rats (I group =  $10^7$  cells). Non-cellular  
therapy was conducted in 10 rats (C group = only medium was  
injected).

30

(Measurement of cardiac function)

Rats were operated on under anesthetization. The  
cardiac function was monitored at Day 14 and 28 after operation.



An ultrasound device (SONOS 5500) with a 12-MHz annular array transducer was used to perform endocardiography (Figure 18). Parasternal short axis imaging and parasternal long axis imaging were performed in the B- and M-imaging modes. In addition to anterior wall pressure, global parameters (e.g., left ventricle end-diastolic diameter, left ventricle endsystolic diameter, fractional shortening, and ejection fraction) were measured (Figure 14).

10 (Histology)

2 and 4 weeks after, the rats were sacrificed with excessive pentobarbital, and the hearts were excised. The hearts were fixed with 10% formaldehyde and embedded in paraffin. Serial 5-mm thick sections were cut from the base to the apex of the heart along a longitudinal axis thereof using a low temperature bath. Thereafter, treatment was performed for standard histology (as shown in Figures 15 and 17, hematoxylin-eosin staining was performed for visualization of muscle, and Masson's Trichrome staining for evaluating collagen content was performed for evaluation of collagen content).

(Results)

25 (Effect of myoblast implantation on cardiac function)

According to the experiments, myocardial infarction resulted in acute mortality of less than 20% within 24 hours after operation. The cell implantation procedure did not cause additional animal death.

30

Ventricle remodeling characteristically caused global enlargement of heart cavity and pump failure. At 2, 4, and 8 weeks after operation, tissue (MS group) and the

MI group had a significant decrease in left ventricular diameter, and both the left ventricular end-diastolic area (LVEDA) and left ventricular endsystolic area (LVESA) were improved after the treatment. Further, the ejection  
5 fraction (EF) value and fractional shortening (FS) value of the myoblast sheet group were also high compared with those of the MI group (Figure 19).

Hearts in the control group which were not subjected  
10 to cell therapy showed further dilation of a ventricle, hypertrophy of an anterior wall, and clearly low ejection fraction (EF) and fractional shortening (FS) values.

(Histological findings)

15 Histology revealed that the impaired hearts of the MI group had a dilated wall having nonuniform thickness (Figure 20), and contained substantially no patches of implanted cells (Figure 21). In contrast, the infarcted heart with the implanted sheet had a wall which did not dilate  
20 and was uniformly thick, and adequately cellularized, and did not have scarring. The survival of the implanted sheet was identified at 2, 4, and 8 weeks after implantation.

Technical loss of implantation cells was analyzed  
25 by RT-PCR based on the presence of the Y chromosome. As a result, the loss was smaller at 15 minutes and 1 day after operation in the MS group than in the MI group ( $3.7 \pm 0.5\%$  vs  $1.7 \pm 0.5\%$  of the total number of heart cells).

30 (Sequential photographs)

By taking a motion picture, it was confirmed that the myoblast implantation of the present invention actually caused pulsation (Figures 22A-F to 29).

Figures 22A to 22F show a motion picture display of a result of an electrophysiological examination, presenting representative frames (still images). Figures 22A to 22C indicate the control, and Figure 22D to 22F indicate results of a myoblast sheet of the present invention.

Figures 23A to 23C show a motion picture display of expression of GFP, presenting representative frames (still images). It is observed that a myoblast sheet of the present invention was actually pulsating.

Figures 24A to 24C show a motion picture display of an ultraechogram of an infarcted heart treated according to the present invention, presenting representative frames (still images).

Figures 25A to 25C also show a motion picture display of an ultraechogram of an infarcted heart treated according to the present invention, presenting representative frames (still images). The left portion of the figure shows a control infarcted heart, while the right portion shows a result of a myoblast sheet of the present invention. As can be seen from the figure, the present invention substantially cured infarct, so that the heart pulsated substantially normally.

Figures 26A to 26C are photographs showing the same sample as in Figures 25A to 25C at different time points. As can be seen from these figures, the infarcted heart was substantially cured by the present invention.

Figures 27A to 27C show a motion picture display of

an ultraechogram of an infarcted heart treated according to the present invention, presenting representative frames (still images). As can be seen from the figure, the infarct was cured by the treatment of the present invention.

5

(RT-PCR)

Next, in order to show cell affinity, the number of implanted cells was quantified by RT-PCR. In this example, the number of gene copies was evaluated by TaqMan assay for SRY and IL2.

10

RT-PCR was performed using a primer which was a portion of a gene derived from the Y chromosome in the male cells. By measuring the amount of the gene in the chromosomes in a female host, affinity was confirmed.

15

The following primer was used.

As a reaction solution, the following PCR mixed reaction solution was used.

20

Universal Mix (provided by the manufacturer)	12.5 $\mu$ l
Primer (100 $\mu$ M) forward	0.05 $\mu$ l
Primer (100 $\mu$ M) reverse	0.05 $\mu$ l
Probe (50 $\mu$ M)	0.1 $\mu$ l
dH <sub>2</sub> O	10.3 $\mu$ l
Template (DNA)	2.0 $\mu$ l

The primer and probe sequences are described below.

SRY (forward primer) GCC TCA GGA CAT ATT AAT CTC TGG AG  
(SEQ ID NO. 15)

5 (reverse primer) GCT GAT CTC TGA ATT CTG CAT GC (SEQ  
ID NO. 16)

(probe) AGG CGC AAG TTG GCT CAA CAG AAT CC (SEQ  
ID NO. 17)

IL2 (forward primer) GCC TTG TGT GTT ATA AGT AGG AGG C (SEQ  
10 ID NO. 18)

(reverse primer) AGT GCC AAT TCG ATG ATG AGC (SEQ ID  
NO. 19)

(probe) TCT CCT CAG AAA TTC CAC CAC AGT TGC  
TG (SEQ ID NO. 20)

15

100 ng of each genomic DNA was used to perform PCR.  
The mixed reaction solution was placed in wells of MicroAmp  
Optical 96-well reaction plates. The wells were capped with  
MicroAmp Optical Caps. Air bubbles were briefly removed,  
20 and the liquid present in the bottom of each well was collected.  
Each gene was measured in triplicate. PCR was performed using  
the ABI Prism 7700 Sequence Detection System.

Standard DNA was the following: (200 ng. 40 ng.  
25 8 ng. 1.6 ng 0.32 ng 0.064 ng); (SRY 200 ng =  $3 \times 10^4$   
copies; IL2 200 ng =  $6 \times 10^4$  copies).

Parameters for thermal cycling were described below.

Stage 1	Stage 2	Stage 3
50°C/2 min	95°C/10 min	95°C/15 sec-60°C/1 min 40 cycles

30

The index of male cells was obtained by the following expression:

$$(2 \times \text{SRY} / \text{IL2}) \times 100.$$

5

As a result, as shown in Figure 28, it was demonstrated that the sheet (three-dimensional tissue) implantation has a more satisfactory level of affinity than that of needle (cell itself) implantation.

10

(Demonstration of cellularity)

What kind of cell type cells contained in an implanted cardiomyocyte sheet were differentiated into, was examined.

15

Therefore, Masson's Trichrome staining, HE (hematoxylin-eosin) staining, MHC fast staining, and MHC slow staining were performed.

These procedures were performed as follows.

20

<Masson's Trichrome Staining>

Masson's Trichrome staining is performed as follows.

Masson's Trichrome staining stains nuclei with iron hematoxylin. Thereafter, small pigment molecules (acid fuchsin, xylidine ponceau) having a high diffusion rate enter cell reticular channels, and next, large pigment molecules (aniline blue) having a low diffusion rate enter the rough structure of collagen fibers, thereby staining the cell with blue.

30

Masson's Trichrome staining uses the following reagents.

## A) Dye mordant

aqueous 10% trichloroacetic acid solution 1 part  
aqueous 10% potassium dichromate solution 1 part

## 5 B) Weigert's iron hematoxylin solution (equal amounts of solution 1 and solution 2 are mixed in use)

## solution 1

hematoxylin 1 g  
100% ethanol 100 ml

10

## solution 2

ferric chloride 2.0 g  
hydrochloric acid (25%) 1 ml  
distilled water 95 ml

15

## C) 1% hydrochloric acid 70% alcohol

## D) I solution

1% Biebrich red 90 ml  
1% acid fuchsin 10 ml  
acetic acid 1 ml

20

## E) II solution

phosphomolybdic acid 5 g  
phosphotungstic acid 5 g  
distilled water 200 ml

25

## F) III solution

aniline blue 2.5 g  
acetic acid 2 ml  
distilled water 100 ml

30

## G) 1% acetic acid water

Procedure for Masson's Trichrome Staining:

1. deparaffinization, washing with water, distilled water;
2. mordanting (10 to 15 min);
- 5 3. washing with water (5 min);
4. Weigert's iron hematoxylin solution (5 min);
5. light washing with water;
6. separation with 1% hydrochloric acid 70% alcohol;
7. color development, washing with water (10 min);
- 10 8. distilled water;
9. I solution (2 to 5 min);
10. light washing with water;
11. II solution (30 min or more);
12. light washing with water;
- 15 13. III solution (5 min);
14. light washing with water;
15. 1% acetic acid/water (5 min);
16. washing with water (quick); and
17. dehydration, clearing, mounting.

20

With Masson's Trichrome staining, collagen fiber, reticular fiber and glomerular basement membrane are stained vividly blue, nuclei are stained black-violet, plasma is stained pale-red, erythrocytes are stained orange-yellow  
25 to deep-red, mucus is stained blue, basophilic granules are stained blue and eosinophilic granules are stained red, and fibrin is stained red. Therefore, a blue-stained area can be calculated as a fibrous site.

30

<Hematoxylin-Eosin (HE) Staining>

The acceptance or vanishment of cells in a sheet was observed by HE staining. The procedure is described as follows. A sample is optionally deparaffinized (e.g., with



pure ethanol), followed by washing with water. The sample is immersed in Omni's hematoxylin for 10 min. Thereafter, the sample is washed with running water, followed by color development with ammonia water for 30 sec. Thereafter, the sample is washed with running water for 5 min and is stained with eosin hydrochloride solution for 2 min, followed by dehydration, clearing, and mounting.

<MHC Fast Staining>

- 10 MONOCLONAL ANTI-SKELETAL MYOSIN (FAST)  
MYOSIN HEAVY CHAIN: MY-32 (skeletal myoblasts)  
Specific reactivity with rats and human
1. Make 5um thick sections from frozen block.
  - 15 2. Sections are fixed in acetone at -20°C for 5-10 mins.  
(Paraffin blocks should be deparaffinized and rehydrated).
  3. Endogenous peroxide activity is blocked in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 mins at RT.
  - 20 (1 ml 30% H<sub>2</sub>O<sub>2</sub> + 99 ml methanol)
  4. Wash with PBS (3 × 5 mins).
  5. Incubate with primary monoclonal antibody (MY-32) in the moist chamber at 4°C for overnight (1 µl antibody + 200 µl PBS per slide).
  - 25 6. Next day wash with PBS (3 × 5 mins).
  7. Apply anti mouse and anti rabbit no. 1 Biotynalated link for 30 mins -1 hrs at RT.  
(apply about 3 drops directly on slide).
  8. Wash with PBS (3 × 5 mins).
  - 30 9. Apply about 3 drops directly Streptavidin HRP no. 2 for LSAB. 10-15 mins.
  10. Wash with PBS (3 × 5 mins).
  11. Apply DAB (5 ml DAB+5 µl H<sub>2</sub>O<sub>2</sub>).

- 134 -

12. Observe under microscope for brownish colour.
13. Dip in water for 5 mins.
14. Apply HE for 30 sec-1 min.
15. Wash few times.
- 5 16. Ion exchange water wash 1 time.
17. 80% ethanol wash for 1 min.
18. 90% ethanol wash for 1 min.
19. 100% ethanol wash for 1 min (3 times).
20. Xylene wash for 1 min (3 times), Coverslip.
- 10 21. Examine color development.

<MHC Slow Staining>

Monoclonal Anti Myosin: (skeletal slow)

Specific reactivity with dog, rats and human

- 15 1. Make 5  $\mu$ m thick sections from frozen block.
2. Sections are fixed in acetone at  $-20^{\circ}\text{C}$  for 5-10 mins.  
(Paraffin blocks should be deparaffinized and rehydrated).
- 20 3. Endogenous peroxide activity is blocked in 0.3%  $\text{H}_2\text{O}_2$  in methanol for 20 mins at RT.  
(1 ml 30%  $\text{H}_2\text{O}_2$  + 99 ml methanol)
4. Wash with PBS (3  $\times$  5 mins).
5. Incubate with primary monoclonal antibody NOQ7 in the moist chamber at  $4^{\circ}\text{C}$  for overnight (1  $\mu$ l antibody + 200  $\mu$ l PBS per slide).
- 25 6. Next day wash with PBS (3  $\times$  5 mins).
7. Apply anti mouse and anti rabbit no.1 Biotynalated link for 30 mins -1 hrs at RT.
- 30 (apply about 3 drops directly on slide).
8. Wash with PBS (3  $\times$  5 mins).
9. Apply about 3 drops directly Streptavidin HRP no. 2 for LSAB. 10-15 mins.

- 135 -

10. Wash with PBS (3 × 5 mins).
11. Apply DAB (5 ml DAB + 5  $\mu$ l H<sub>2</sub>O<sub>2</sub>).
12. Observe under microscope for brownish colour.
13. Dip in water for 5 mins.
- 5 14. Apply HE for 30 sec-1 min.
15. Wash few times.
16. Ion exchange water wash 1 time.
17. 80% ethanol wash for 1 min.
18. 90% ethanol wash for 1 min.
- 10 19. 100% ethanol wash for 1 min (3 times).
20. Xylene wash for 1 min (3 times), Coverslip.
21. Examine color development.

15 As a result, as shown in Figure 29, it was demonstrated that the implanted myoblast sheet was differentiated from fast fiber to slow fiber. These markers can be used to identify a three-dimensional tissue structure of the present invention.

#### 20 (Conclusion)

Skeletal myoblast implantation attenuated heart remodeling and regenerated impaired myocardium to ameliorate global cardiac function as compared with cell implantation. This finding suggests a promising strategy for regenerative  
25 therapy for myocardium.

Therefore, it was demonstrated that when a myoblast sheet was implanted as a three-dimensional structure, the cardiac function of impaired myocardium was ameliorated.  
30 In this example, by using direct injection, autologous skeletal myoblast (SM) implantation was clinically applied. Loss of implanted cells and implanted extracellular matrix (ECM) due to direct injection limits the number of skeletal

myoblasts and the ability of skeletal myoblasts.

Concerning clinical tests using an autologous cell source, autologous myoblasts are preferably used. It was  
5 also demonstrated that tissue implantation is more advantageous to regenerate an injured heart than cell implantation.

(Example 3: Skeletal myoblast - Implantation of  
10 tissue-engineered myoblast sheet improves cardiac function with attenuation of cardiac remodeling in cardiomyopathic hamsters)

Next, it was examined whether or not a prosthetic tissue or three-dimensional structure produced using  
15 skeletal myoblasts ameliorates cardiomyopathy.

Cell therapy is a promising strategy for ischemic cardiomyopathy. However, direct injection methods seem to  
have limitations for generalized cell delivery in dilated  
20 cardiomyopathy (DCM). Given this body of evidence, the present inventors considered that a tissue-engineered myoblast sheet implantation might be a superior and promising method to ameliorate the cardiac function in DCM. Therefore, the present inventors carried out this example.

25

(Method)

Male 27-week old BIO TO-2 (dilated cardiomyopathy (DCM)) hamsters which showed moderate cardiac remodeling were used as recipients. Myoblasts isolated from BIO FIB  
30 hamsters (FIB) were cultured on dishes grafted with a temperature-responsive polymer made of poly(N-isopropylacrylamide), and detached as a cell sheet at 20°C without enzymatic treatment.

Three different therapies were conducted: (1) myoblast sheet implantation group (S group, n=8); (2) myoblast (isolated from FIB) injection group (T group, n=10);  
5 and (3) Sham operation group (C group, n=10). In the S group, a myoblast sheet was implanted on the left ventricular (LV) wall. In the T group, myoblasts were injected into the right ventricular (RV) wall and the left ventricular (LV) wall.

10 (Results)

(Functional recovery of infarcted myocardium)

B-mode analysis demonstrated that the dilation of a left ventricle was adequately suppressed and global wall motion was adequately preserved in the T group as compared  
15 with the C group (Figure 18).

After the myoblast sheet was implanted, an ultrasound echogram demonstrated that dilated LV dimension was significantly reduced, whereas the hearts in T and C groups  
20 showed a progression of left ventricular (LV) dilation (Figure 33A). Six weeks after operation, fractional shortening (FS) was significantly improved in the S and T groups compared with that in the C group. After seven weeks, FS in the S group was maintained at the preoperative level,  
25 while FS in the other groups decreased gradually. Although the max velocity of the mitral valve E-wave in the S group dropped slightly one week after the sheet implantation, this was recovered to the preoperative level 2 weeks after operation. The averaged E-wave in the S and T groups was  
30 significantly higher than that in the C group 4 weeks after operation and thereafter. Histological examinations in the S group demonstrated that the implanted sheet almost covered the whole heart and the left ventricle (LV) wall thickness

was increased with surviving myoblasts (Figures 30A to 30D). As can be seen from Figure 30B, the implanted myoblasts, which constituted a tissue structure, were intimately attached to and accepted by the heart to aid the cardiac function. Figures 30C and 30D clearly show that expression of  $\alpha$ -sarcoglycan and  $\beta$ -sarcoglycan had a score of 2 to 3 and about 3, respectively, i.e., the heart was substantially moderately recovered, where normal expression has a score of 5 and DCM hamsters have a score of about 3. It is known that DCM hamsters have a symptom of partial dilated cardiomyopathy caused by a decrease in expression of sarcoglycan. It was demonstrated that a three-dimensional tissue structure of the present invention has an effect of supplementing such gene expression.

Figure 33B clearly shows that use of the three-dimensional tissue structure of the present invention allowed DCM hamsters to survive beyond 48 weeks. This is a significant and unexpected effect compared with injection (only cells) or control (in either case, all DCM hamsters died after 38 to 40 weeks). As shown in Figure 33C, the contractility of the left ventricle with dilated cardiomyopathy was significantly ameliorated. This result shows that the structure of the present invention can be applied to hearts. The result corresponds to about a 25-year extension of life span, demonstrating a significant effect of the three-dimensional tissue structure of the present invention. The average life-span of the DCM hamsters was about 40 weeks, while the longest life-span of the DCM hamsters having an implanted sheet was about 70 weeks. Assuming that the life-span of a normal hamster is about 2 years and the life-span of a human is 80 years, the life-span of the implanted hamster was prolonged by about 30 weeks which is equivalent

to about 25 years in a human. Thus, it was demonstrated that the three-dimensional structure of the present invention has a significant effect on cardiomyopathy.

5 (Conclusion)

Myoblast sheet implantation reduced the progression of heart hypertrophy with improvement of cardiac function in dilated cardiomyopathic (DCM) hearts. Myoblast sheet implantation may be a promising method to restore the cardiac  
10 function with attenuation of cardiac remodeling in DCM hearts.

(Example 4: Therapy for infarct pig model)

In this example, aiming for more clinical findings, skeletal myoblast sheets were implanted into a larger animal  
15 model of myocardial infarct to study amelioration of cardiac function.

(Method)

20 A thoracotomy was performed on 30 kg pigs under general anesthetization and the LAD was ligated to produce myocardial infarct models. Three different therapies were conducted: 1) skeletal myoblast sheet group; 2) skeletal myoblast injection group; and 3) control group. For these  
25 groups, changes in cardiac function and myocardial tissue were examined (Figure 34). Skeletal myoblasts were collected from autologous thigh muscle. Collagenase, trypsin EDTA, gentamicin sulfate, and amphotericin B were prepared and filtered through a 0.22- $\mu$ m filter to formulate  
30 a dissociating solution. SkBM Basal Medium, bovine fetal serum, EGF, sodium dexamethasone phosphate, gentamicin sulfate, and amphotericin B were prepared and filtered through a 0.22  $\mu$ m filter to formulate primary culture medium.

The dissociated cells were cultured on dishes grafted with a temperature responsive macromolecule made of poly(N-isopropylamide) and were detached as a cell sheet by changing temperature instead of enzymatic treatment.

5

#### (Results)

The group with cell sheet implantation had ameliorated cardiac functions, i.e., contractility and expansibility (Figures 35 and 36). In addition, it was confirmed that the implanted cells were accepted by the myocardial infarct portion.

10

#### (Conclusion)

In experiments using animals other than rodents, it was confirmed that a therapy using a myoblast sheet of the present invention had an effect of ameliorating cardiac function.

15

#### (Example 5: Synovial cells)

To demonstrate an effect of the present invention in the case of another type of cell, a sheet of synovial cells (cells containing tissue stem cells) was prepared and implanted into myocardial infarct models to examine an effect of ameliorating cardiac function.

20

25

#### (Methods)

Knee joints were removed from 8-week old rats. Synovial tissue was cut off the internal surface of the joint. The tissue was attached to culture medium containing 20% FCS and DMEM high glucose for cell culture. The resultant cells were cultured on dishes grafted with a temperature responsive macromolecule made of poly(N-isopropylamide) and were detached as a cell sheet by changing temperature instead

30



of enzymatic treatment. For myocardial infarct models prepared by LAD ligation, three different therapies were conducted: 1) cell sheet group; 2) cell injection group; and 3) control group. For these groups, changes in cardiac function and myocardial tissue were examined.

(Results)

The group with cell sheet implantation had ameliorated cardiac functions, i.e., contractility and expansibility. In addition, it was confirmed that the implanted cells were accepted by the myocardial infarct portion.

(Conclusion)

In the case of cells differentiated from cell obtained from synovial tissue, it was confirmed that a sheet of such cells had an effect of ameliorating cardiac function.

(Example 6: Stem cells)

As an example of undifferentiated cells, there is a mouse embryonic stem cell known to be capable of differentiating into a cardiomyocyte. In this example, a sheet of such cardiomyocytes was prepared and implanted into myocardial infarct models to study the effect on ameliorating cardiac function.

(Methods)

A resistance gene was introduced into a promoter site of a gene expressing MHC in mouse embryonic stem cells. The cells were cultured in a selective culture containing a high concentration of a drug in which differentiated cells other than cardiomyocytes are killed, so that cardiomyocytes were selected. The resultant cells were cultured on dishes

grafted with a temperature responsive macromolecule made of poly(N-isopropylamide) and were detached as a cell sheet by changing temperature instead of enzymatic treatment. For myocardial infarct models prepared by LAD ligation, three  
5 different therapies were conducted: 1) cell sheet group; 2) cell injection group; and 3) control group. For these groups, changes in cardiac function and myocardial tissue were examined.

10 (Results)

The group with cell sheet implantation had ameliorated cardiac functions, i.e., contractility and expansibility. In addition, it was confirmed that the  
15 implanted cells were accepted by the myocardial infarct portion.

(Conclusion)

In the case of cells differentiated from cells obtained from synovial tissue, it was confirmed that a sheet  
20 of such cells had an effect of ameliorating cardiac function.

(Example 7: Preparation of prosthetic tissue using ascorbic acid)

Next, an influence of ascorbic acid or a derivative  
25 thereof on production of a prosthetic tissue was studied.

After an adequate amount of myoblasts were produced,  $5 \times 10^6$  cells were cultured on 10-cm temperature responsive culture dishes. For culture, SkBM Basal Medium (Clonetics  
30 (Cambrex)) was used. Next, ascorbic acid 2-phosphate (0.5 mM), magnesium ascorbic acid 1-phosphate (0.1 mM), and sodium L-ascorbate (0.1 mM) were added to the medium. 4 days after culture start, the cells were detached at 20°C. As

a control, a prosthetic tissue was prepared in a culture system without ascorbic acids.

(Results)

5           When ascorbic acid was added, the prosthetic tissue was detached much more easily than that in the culture system without any ascorbic acids. In addition, tissue was not cultured to a size of several millimeters in the culture system without ascorbic acids. If the tissue exceeded such  
10 a size, cracks occurred and the growth stopped. It was also substantially difficult to detach the tissue. Thus, implantable prosthetic tissue could not be provided. In contrast, a prosthetic tissue of the present invention, which was cultured in a medium supplemented with an ascorbic acid,  
15 grown into an implantable size, and was easy to detach. In addition, the prosthetic tissue was isolated while substantially no hole or scar was generated. Inspection of biological connections demonstrated that extracellular matrix-mediated interaction was significant (Figures 37 to  
20 39).

(Example 8: Effect of addition of ascorbic acid 2-phosphate)

25           Next, an influence of ascorbic acid 2-phosphate on production of a prosthetic tissue was studied.

30           After an adequate amount of synovial cells or myoblasts were produced,  $5 \times 10^6$  cells were cultured on 10-cm temperature responsive culture dishes. For culture, SkBM Basal Medium (myoblasts) containing ascorbic acid 2-phosphate (1 mM) or DMEM (synovial cells) containing ascorbic acid 2-phosphate (1 mM) was used. As a control, a prosthetic tissue was prepared in a culture system having

the same medium without ascorbic acids or in another culture system having the same medium with ascorbic acid 1-phosphate (1 mM).

5                   9 days after culture start, the tissue was detached and contracted. The tissue was contracted by a factor of about 3.

10                   The contracted tissue was histologically analyzed by HE staining or the like (Figure 42, synovial cells). It was found that the cells constructed 10 or more layers and the matrix was in the form of collagen mesh or sponge. The matrix had rigidity such that the matrix could be easily pinched with forceps.

15

(Stress-distortion examination (tensile test))

20                   To confirm strength, a tensile test was conducted to obtain a load-time (stress distortion) curve where a sample is stretched. According to this curve, the limit of proportionality, modulus of elasticity, yield point, maximum strength, rupture point, elastic energy, and tenacity are obtained.

(Creep characteristics (indentation test))

25                   Indentation test, which measures creep characteristics, was carried out by determining viscoelasticity. It is possible to observe a phenomenon in which distortion is increased. An instrument, such as a rod or the like, is pushed into a gel material, and the deformation  
30 of the material is monitored.

(Results)

A prosthetic tissue can be detached much more easily

when ascorbic acid 2-phosphate is added than in a culture system without ascorbic acids and in a culture system which contains commonly used ascorbic acid 1-phosphate. In addition, tissue was not cultured to a size of several millimeters in the culture system without ascorbic acids. If the tissue exceeded such a size, cracks occurred and the growth stopped. The size, strength, and the like are greater in the culture system with ascorbic acid 2-phosphate than in the culture system containing commonly used ascorbic acid 1-phosphate. In the system without ascorbic acids, it was also substantially difficult to detach the tissue. Thus, implantable prosthetic tissue could not be provided.

Particularly, the prosthetic tissue cultured in the system containing ascorbic acid 2-phosphate had rigidity such that it can be pinched with forceps. Prosthetic tissues cultured in other systems had less rigidity than that of the prosthetic tissue cultured in the ascorbic acid 2-phosphate-containing system. The prosthetic tissue cultured in ascorbic acid 2-phosphate-containing medium was grown to an implantable size and was easy to detach. In addition, the prosthetic tissue was isolated while substantially no hole or scar was generated. Inspection of biological connection demonstrated that extracellular matrix-mediated interaction was significant.

(Example 9: Effect of prosthetic tissue cultured in the presence of ascorbic acids)

The prosthetic tissue produced in the presence of ascorbic acids in Examples 7 and 8 was implanted into dilated cardiomyopathy hamsters. All the implanted mice were cured, and survived as long as ordinary hamsters do. Therefore, it was demonstrated that the present invention can cure

diseases, which are conventionally believed to be refractory, by providing a specific three-dimensional promoting agent.

(Example 10: Combined therapy)

5           A combined therapy of a sheet as prepared in the above-described examples, and gene therapy, was carried out. The combined therapy aims for promoting angiogenesis in sheet implanted sites, promoting acceptance of implanted sheets, and suppressing cell necrosis within sheets.

10

(Methods)

          A complex of Sendai virus (HVJ) and a liposome was prepared in accordance with the description of a document (Kaneda Y., Iwai K., Uchida T., Increased Expression of DNA  
15   Co-introduced with Nuclear Protein in Adult Rat Liver, Science, 1989, 243:375-378). Hereinafter, this procedure will be briefly described. 200  $\mu$ l of DNA solution was prepared and shaken for 30 sec. The solution was allowed to stand in a 37°C constant temperature bath for 30 sec. This  
20   procedure was performed 8 times. The solution was subjected to ultrasonic treatment for 5 sec, and shaken for 30 sec. 0.3 mL of BSS was added to the solution, followed by shaking in a 37°C constant temperature bath. Inactivated HVJ was added to the solution which was in turn placed on ice for  
25   10 min. The solution was shaken in a 37°C constant temperature bath for 1 hour. 1 mL of 60% sucrose solution and 6 mL of 30% sucrose solution were layered in an ultracentrifugation tube. HVJ liposome solution was placed on the layered solution. BSS was added to the tube. The  
30   tube was ultracentrifuged at 62,800 $\times$ g 4 times for 1.5 hours. A layer immediately above the 30% sucrose solution layer was removed and preserved at 4°C. This liposome was used for gene introduction.

About 0.2 mL of Sendai virus liposome-plasmid complex (containing 15 µg of human HGF cDNA) was injected into a myocardial infarct region. In a control group, an empty vector was used for gene introduction into infarcted myocardium. The human HGF concentration of heart tissue was measured by enzyme-linked immunosolvent assay (ELISA) using an anti-human HGF monoclonal antibody (Institute of Immunology, Tokyo, Japan)) (Ueda H., Sawa Y., Matsumoto K. et al., Gene Transfection of Hepatocyte Growth Factor Attenuates Reperfusion Injury in the Heart, Ann. Thorac. Surg., 1999, 67:1726-1731). The resultant cells were cultured on dishes grafted with a temperature responsive macromolecule made of poly(N-isopropylamide) and were detached as a cell sheet by changing temperature instead of enzymatic treatment. For myocardial infarct models prepared by LAD ligation, three different therapies were conducted: 1) cell sheet group; 2) gene therapy group; 3) combined therapy group; and 4) control group. For these groups, changes in cardiac function and myocardial tissue were examined.

#### (Results)

The cell sheet implantation group and the combined therapy group have ameliorated cardiac functions, i.e., contractility and expansibility. In addition, it is confirmed that in the combined therapy group, angiogenesis is observed and the implanted cells are accepted by the myocardial infarct portion.

#### (Conclusion)

A combination of sheet tissue and gene therapy has an effect of amelioration of cardiac function, an effect

of angiogenesis, and an effect of cell protection. It is also confirmed that cardiac function is further ameliorated.

Although certain preferred embodiments have been  
5 described herein, it is not intended that such embodiments  
be construed as limitations on the scope of the invention  
except as set forth in the appended claims. Various other  
modifications and equivalents will be apparent to and can  
be readily made by those skilled in the art, after reading  
10 the description herein, without departing from the scope  
and spirit of this invention. All patents, published patent  
applications and publications cited herein are incorporated  
by reference as if set forth fully herein.

#### 15 INDUSTRIAL APPLICABILITY

The present invention provides a radical therapeutic  
method, a technique, and a medicament for diseases which  
are difficult to treat by conventional therapies  
20 (particularly, heart diseases exhibiting severe heart failure,  
etc.).



## CLAIMS

1. A three-dimensional structure applicable to heart,  
comprising a cell derived from a part other than myocardium  
5 of an adult.
2. A structure according to claim 1, wherein the cell is  
a stem cell or a differentiated cell.
- 10 3. A structure according to claim 1, wherein the cell is  
a mesenchymal cell.
4. A structure according to claim 1, wherein the cell is  
derived from a myoblast.  
15
5. A structure according to claim 4, wherein the myoblast  
is a skeletal myoblast.
6. A structure according to claim 1, wherein the cell is  
20 a fibroblast.
7. A structure according to claim 1, wherein the cell is  
a synovial cell.
- 25 8. A structure according to claim 1, wherein the cell is  
derived from a stem cell.
9. A structure according to claim 1, wherein the cell is  
derived from a subject, the structure being applied to the  
30 subject.
10. A structure according to claim 1, wherein the cell is  
not derived from a subject, the structure being applied to

the subject.

11. A structure according to claim 1, wherein the structure expresses at least one non-adult heart marker selected from the group consisting of myosin heavy chain IIa, myosin heavy chain IIb, myosin heavy chain IId(IIx), CD56, MyoD, Myf5, and myogenin.

12. A structure according to claim 11, wherein an expression level of the non-adult heart marker in the structure is at least 50% of an expression level of the non-adult heart marker in skeletal myoblasts.

13. A structure according to claim 1, wherein the three-dimensional structure expresses all of myosin heavy chain IIa, myosin heavy chain IIb, myosin heavy chain IId(IIx), CD56, MyoD, Myf5, and myogenin.

14. A structure according to claim 13, wherein an expression level of each of myosin heavy chain IIa, myosin heavy chain IIb, myosin heavy chain IId(IIx), CD56, MyoD, Myf5, and myogenin in the structure is at least about 50% of an expression level thereof in skeletal myoblasts.

15. A structure according to claim 13, wherein an expression level of each of myosin heavy chain IIa, myosin heavy chain IIb, myosin heavy chain IId(IIx), CD56, MyoD, Myf5, and myogenin in the structure is at least about 100% of an expression level thereof in skeletal myoblasts.

16. A structure according to claim 1, wherein the cell derived from a part other than myocardium is a cell not derived from heart.

17. A structure according to claim 1, wherein the applicability to heart includes applicability to myocardium.

5 18. A structure according to claim 1, comprising a monolayer cell sheet.

19. A structure according to claim 1, comprising a multilayer cell sheet.

10

20. A structure according to claim 19, wherein the multilayer cell sheet has biological connection.

15 21. A structure according to claim 20, wherein the biological connection is selected from the group consisting of connection via extracellular matrix, electrical connection, and connection without scaffold.

20 22. A medicament, comprising a three-dimensional structure according to any one of claims 1 to 21.

23. A medicament according to claim 22, wherein the heart has a disease or disorder selected from the group consisting of heart failure, ischemic heart disease, myocardial infarct, cardiomyopathy, myocarditis, hypertrophic cardiomyopathy, 25 dilated phase hypertrophic cardiomyopathy, and dilated cardiomyopathy.

24. A method for producing a three-dimensional structure applicable to heart comprising a cell derived from a part other than myocardium of an adult, the method comprising the steps of:

a) culturing the cell derived from the part other

than myocardium of an adult on a cell culture support grafted with a temperature responsive macromolecule having an upper limit critical solution temperature or lower limit critical solution temperature to water of from 0°C to 80°C;

5           b) setting a culture medium temperature to the upper limit critical solution temperature or more or the lower limit critical solution temperature or less; and

          c) detaching the cultured cell as a three-dimensional structure.

10

25. A method according to claim 24, wherein a treatment using a protein degrading enzyme is not performed in or before the detaching step.

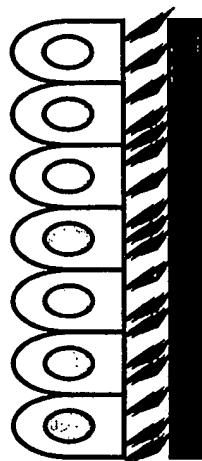
15

26. A method according to claim 24, wherein the temperature responsive macromolecule is poly(N-isopropylacrylamide).

FIG. 1A

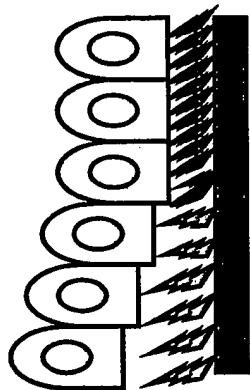
Cell Sheet Constructs

A



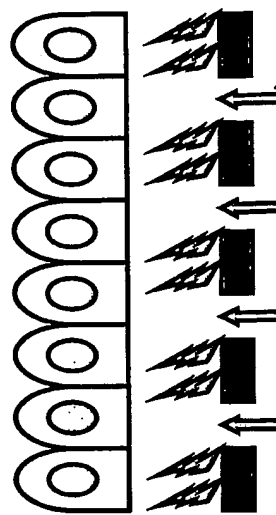
Hydrophobic PIPAAm  
Remains attached: 37°C

B



Intermediate stage  
Partial detachment: 20°C

C

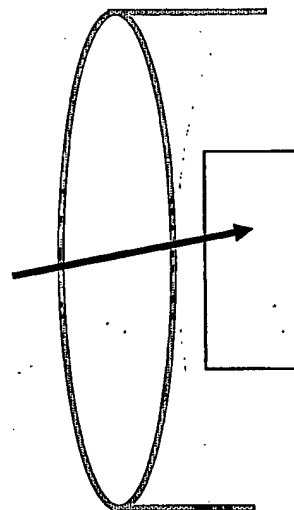


Hydrophilic PIPAAm  
Completely detached sheet

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Temperature responsive polymer

Poly (N-isopropylacrylamide) (PIPAAm)



Poly (acrylamide) (PAAm)

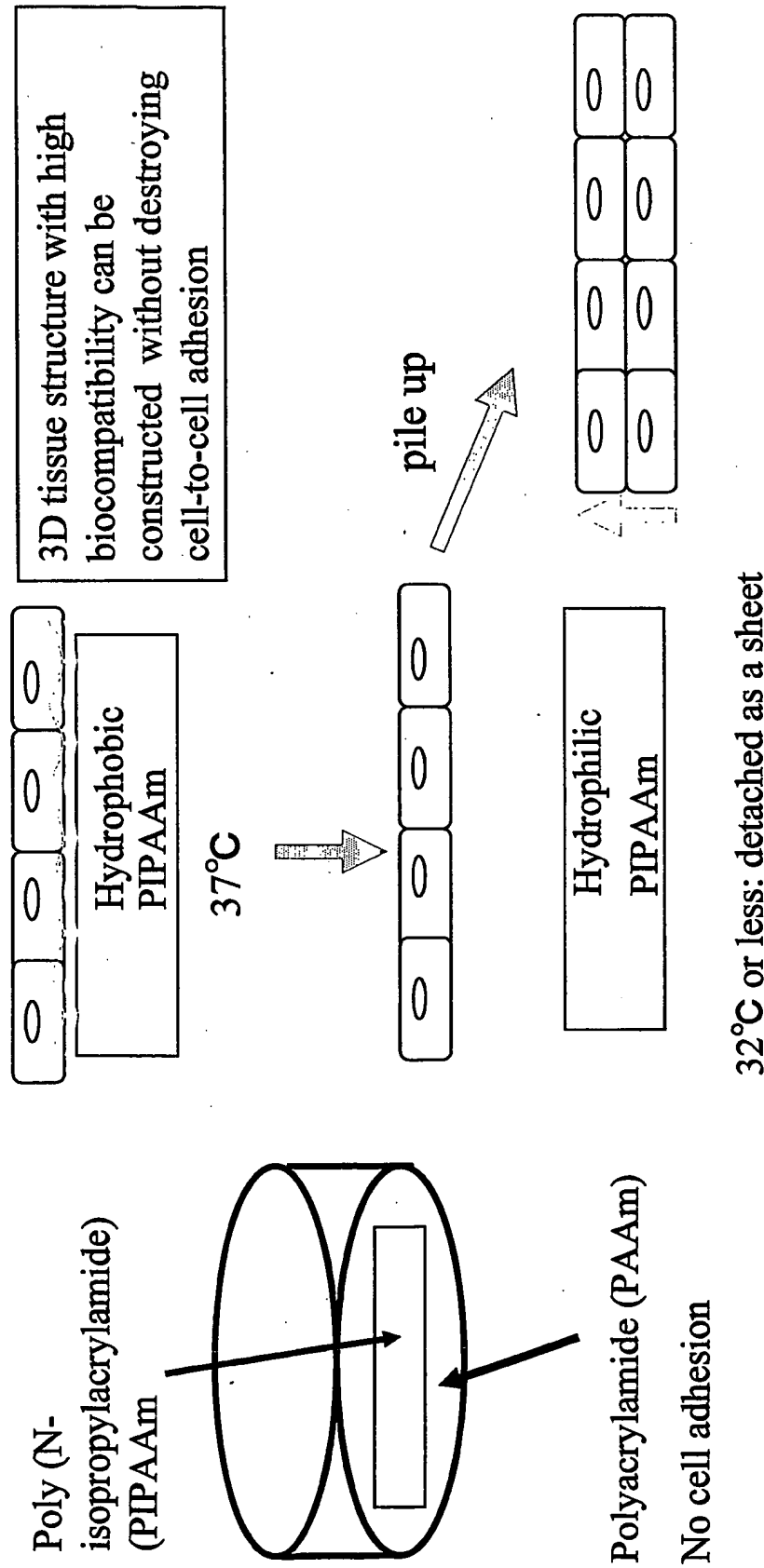
Exemplary Dimensions:

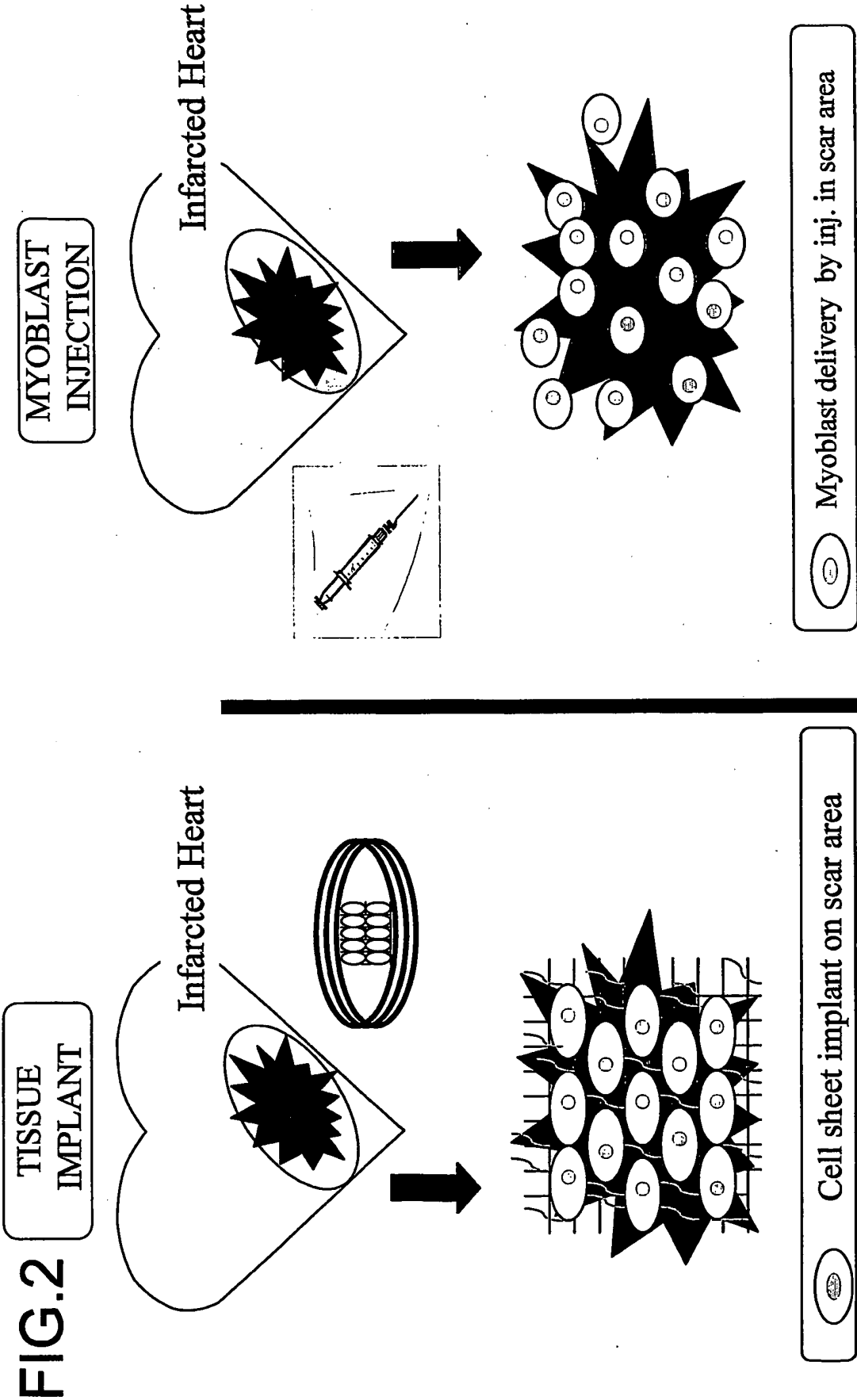
$1.11 \pm 0.05 \text{ cm}^2$  in area

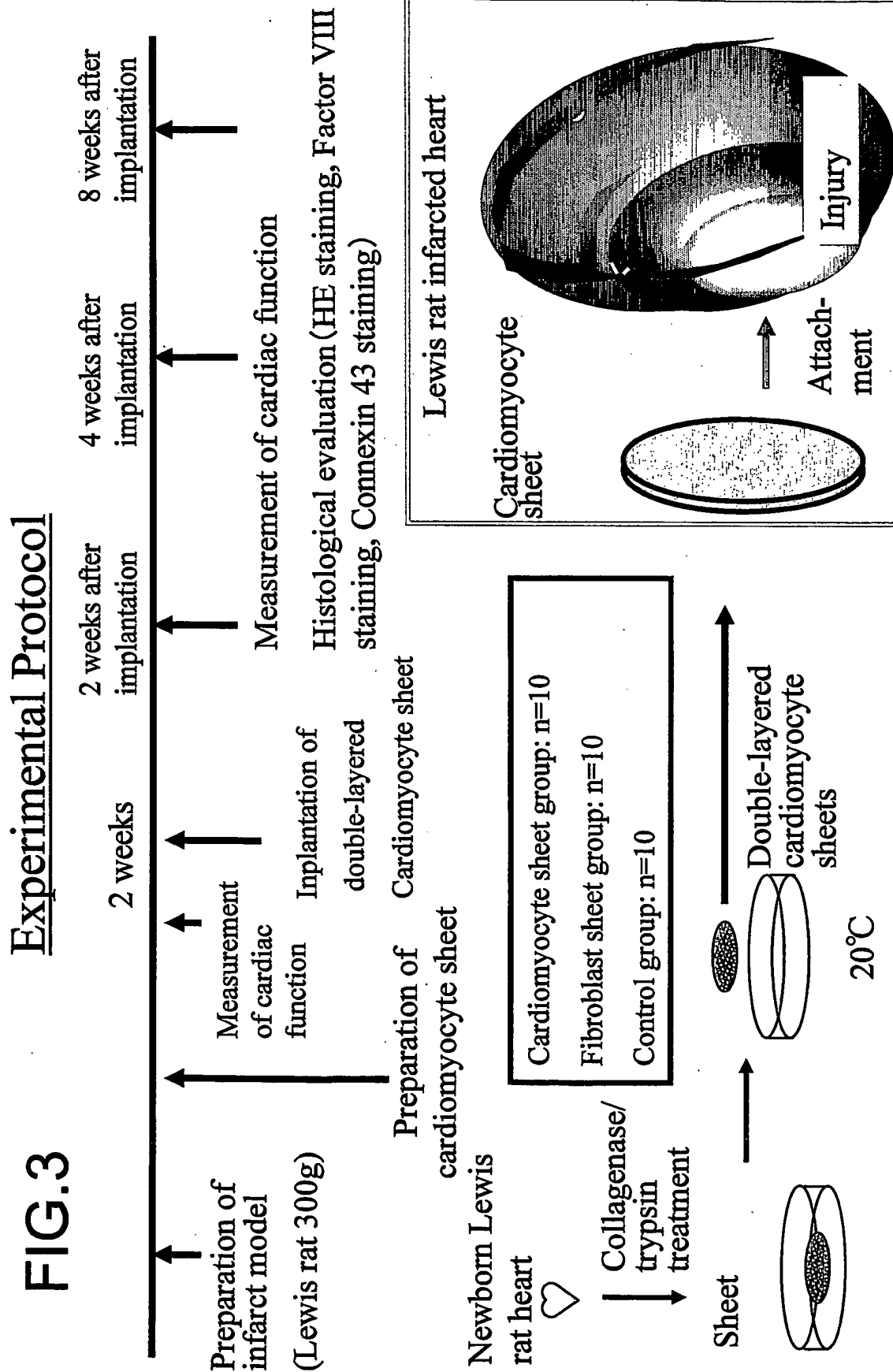
$50.2 \pm 6.0 \mu\text{m}$  thick

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FIG. 1B

Temperature responsive culture dish

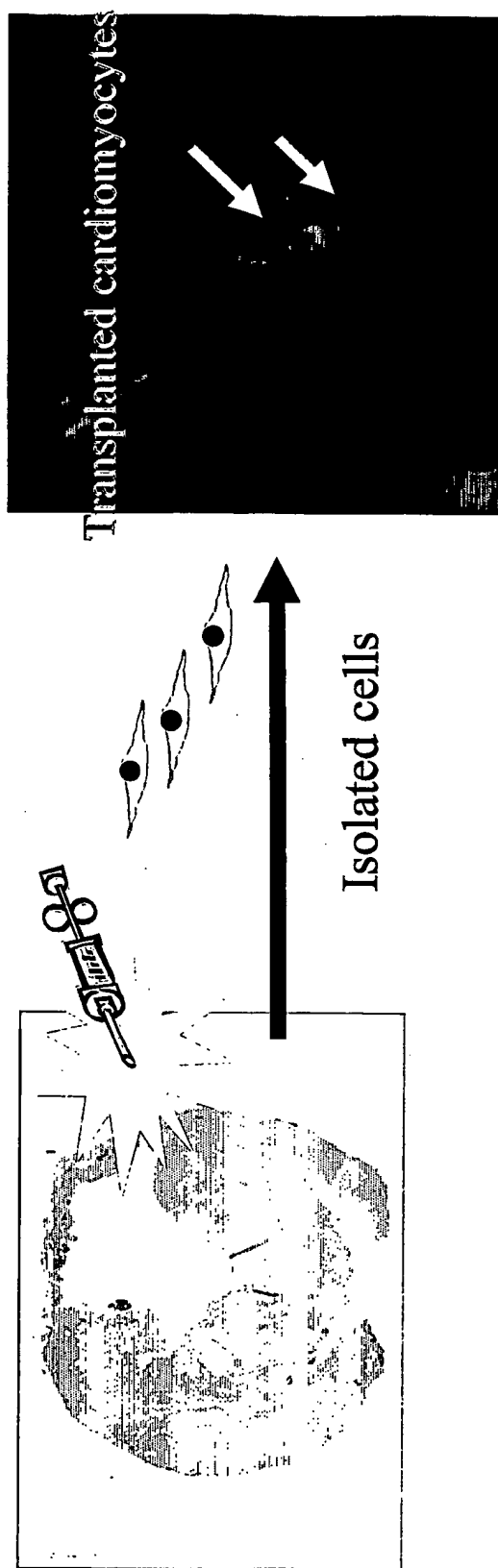






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**FIG.4** Regenerative therapy for cardiac  
muscle by cell transplantation



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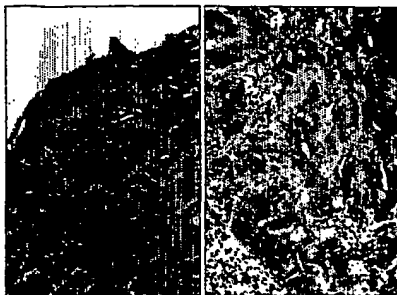
## FIG.5 Problems with tissue transplantation

### Cardiac muscle graft with scaffold

Alignment and cell-to-cell adhesion of transplanted cells within scaffold

Changes in scaffold in organism: elicitation of inflammation

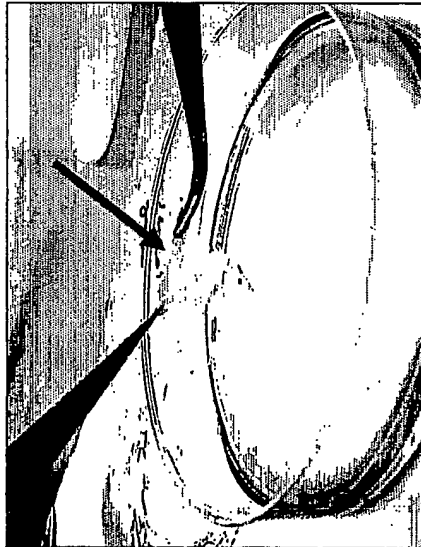
Acceptance of scaffold by recipient's heart



Development of high biocompatible cardiac muscle graft without scaffold

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FIG. 6

Implantation of cardiomyocyte sheet into infarcted heart

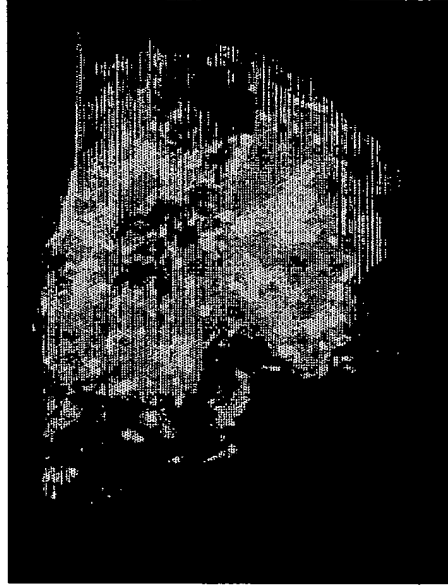
Cardiomyocyte sheet



Implantation into rat infarct model

*In vitro*

Implantation of GFP rat newborn cardiomyocyte sheet

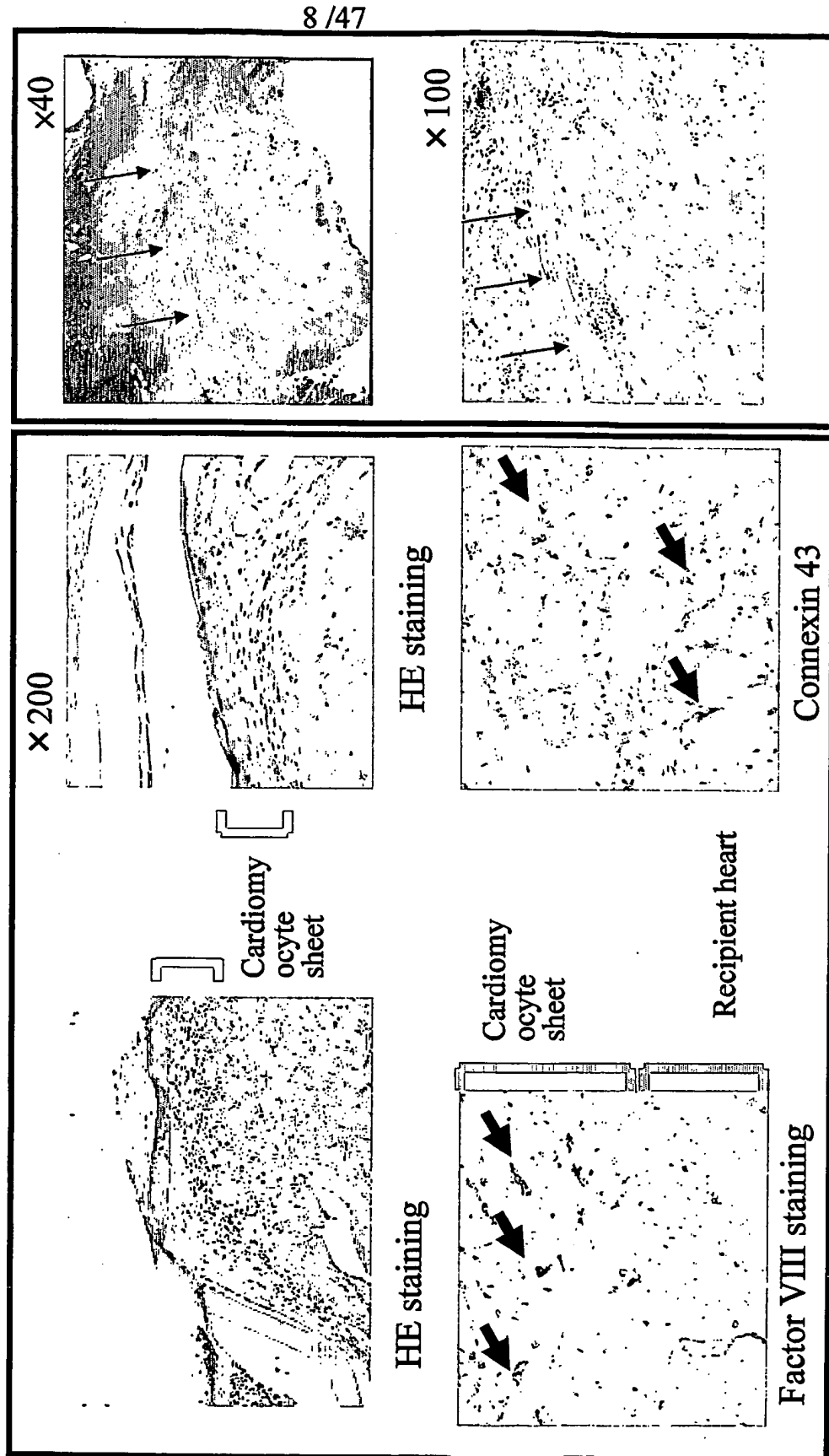
*In vivo*

**FIG.7**

Tissue

2 weeks after implantation

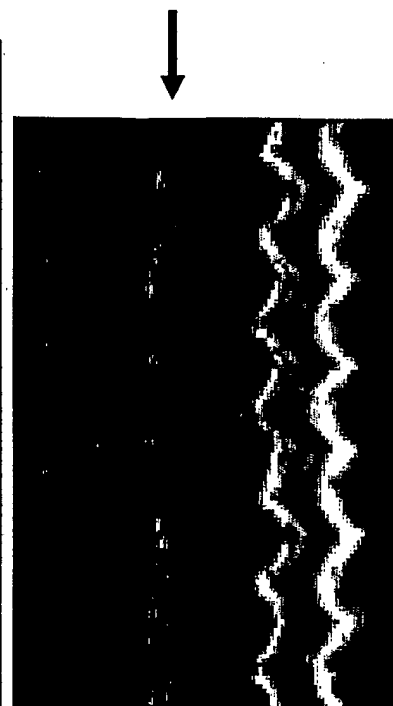
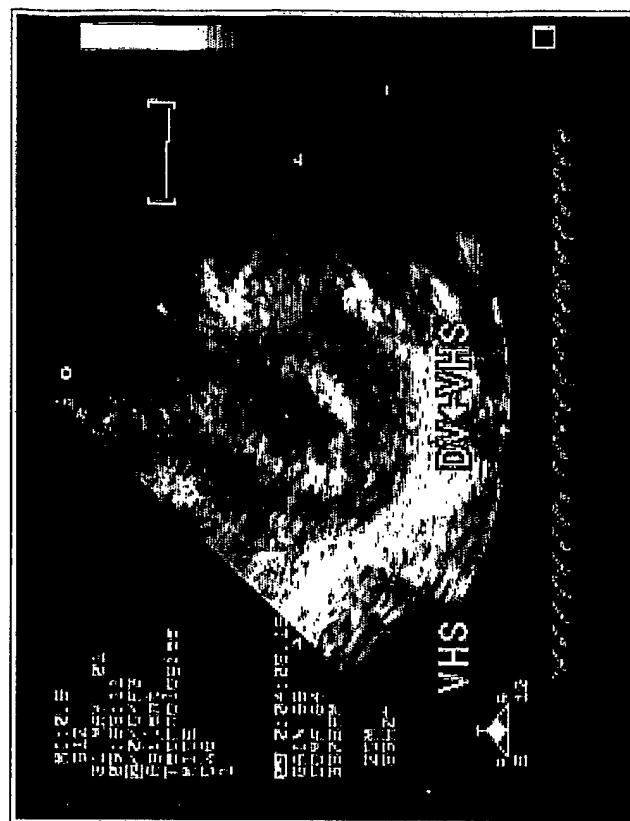
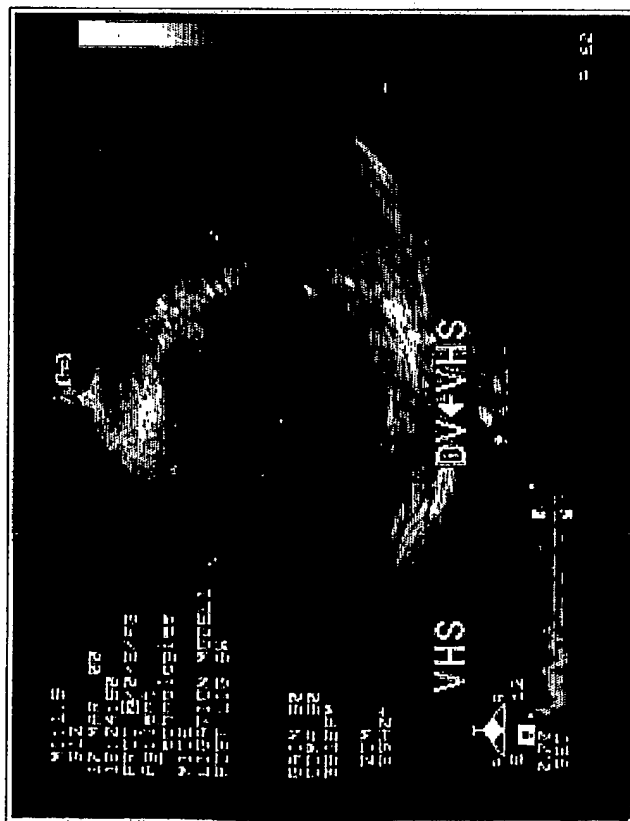
8 weeks after  
implantation



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# Control

## Implantation of prosthetic tissue



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**FIG.9** Evaluation of cardiac function - 2

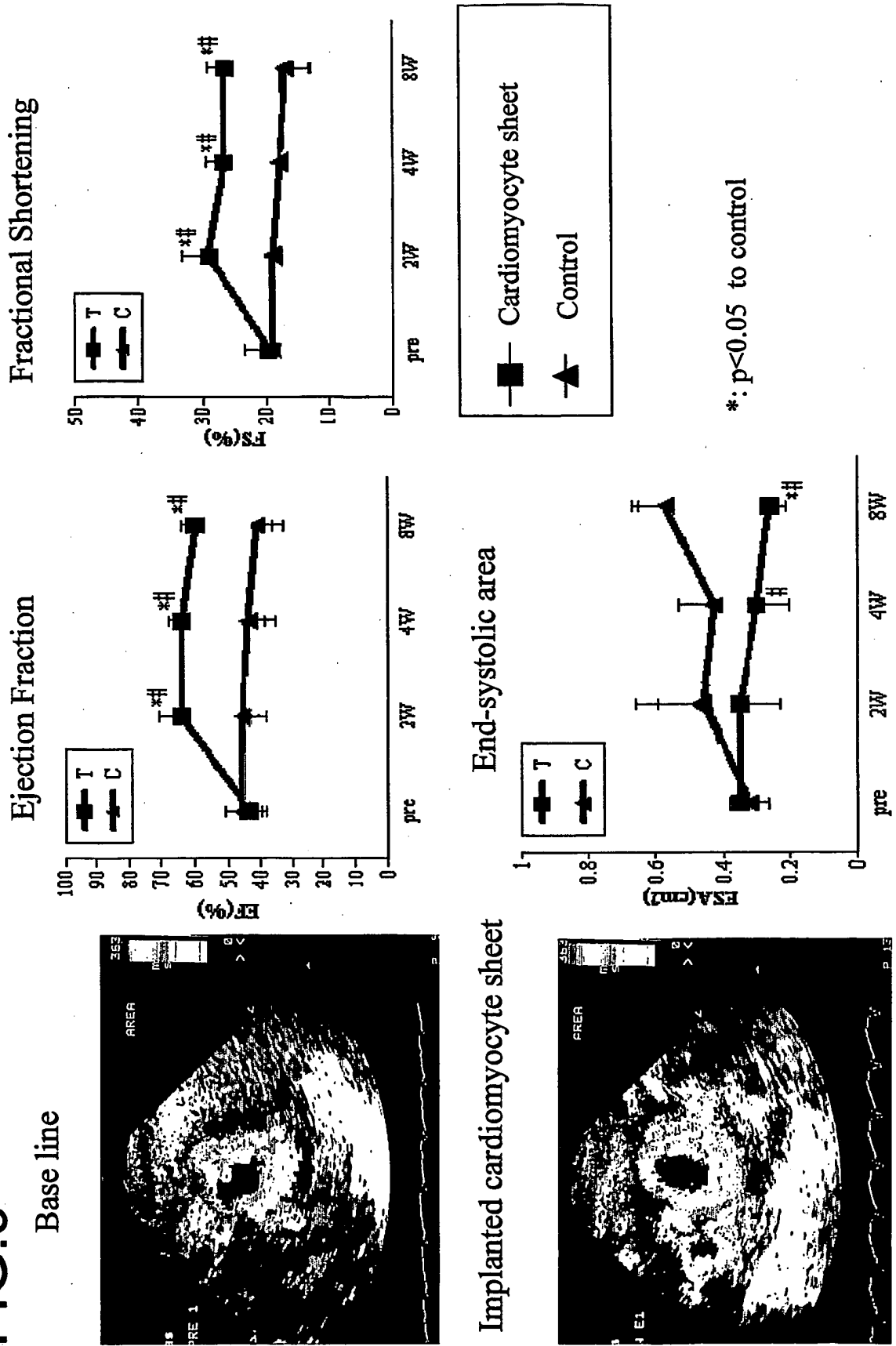


FIG.10

Electrophysiological Evaluation

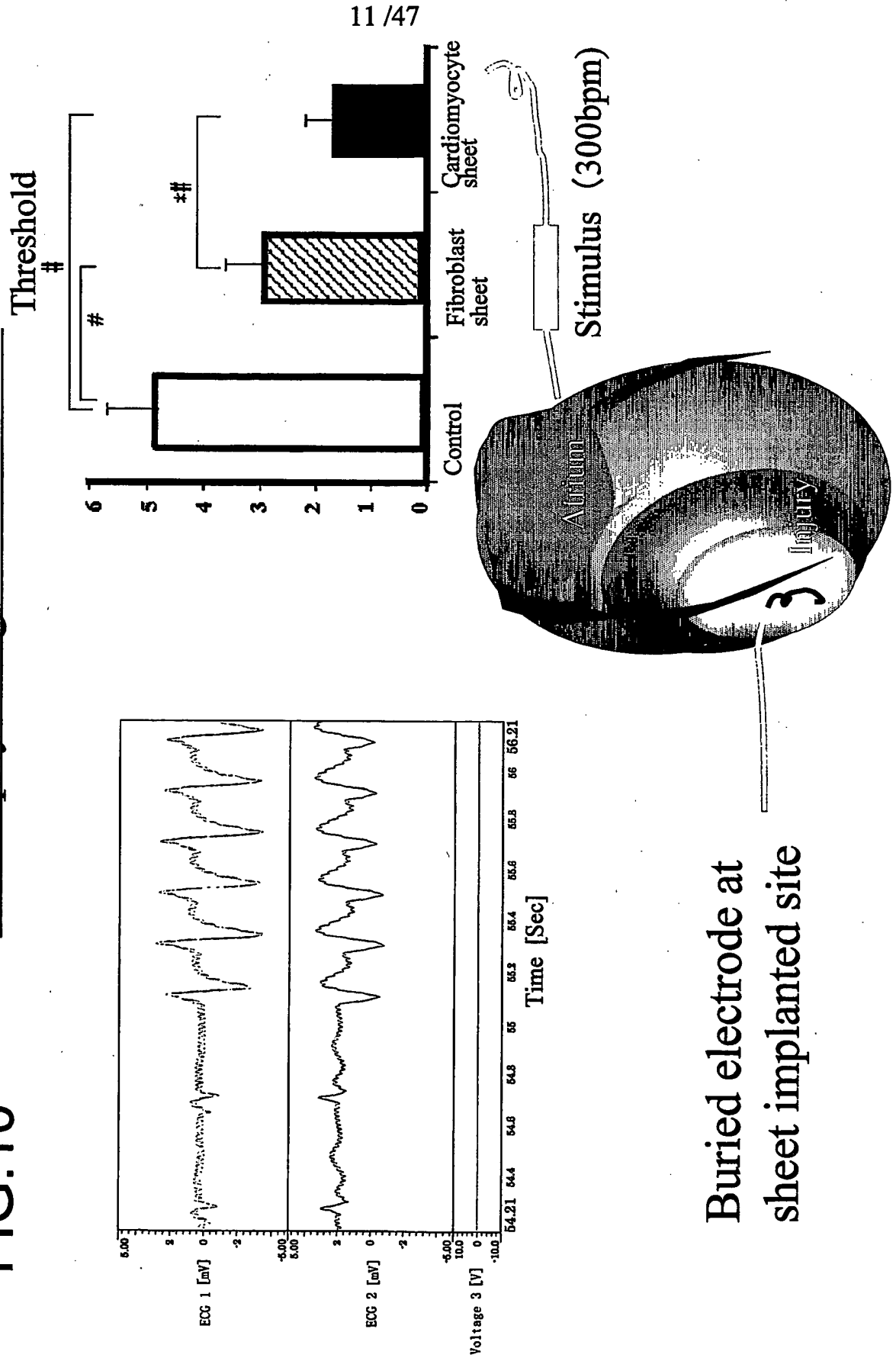
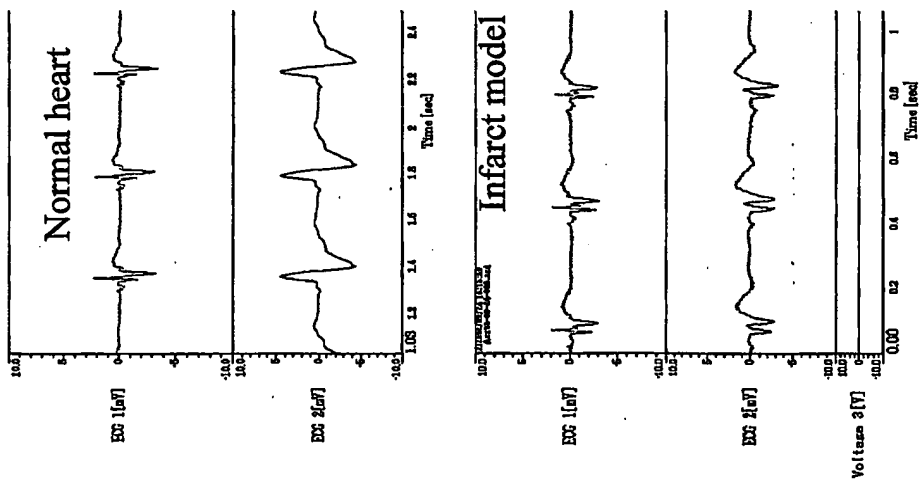


FIG.11



ECG 1: ECG (Surface)  
 ECG 2: Normal heart (anterior wall)  
 Ligation model (injured)  
 Prosthetic tissue implanted  
 (prosthetic tissue injured)

# Electrophysiological Evaluation

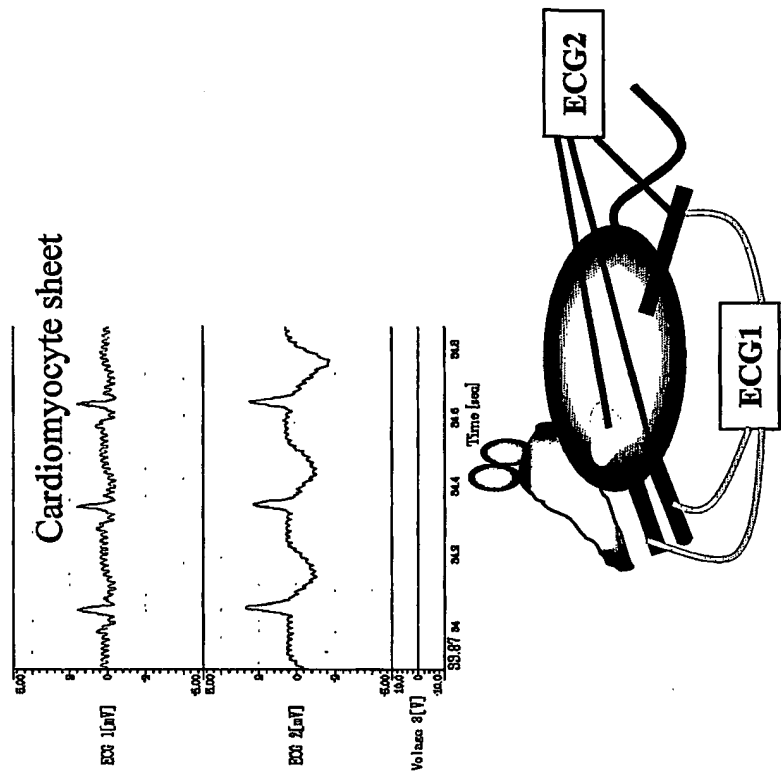


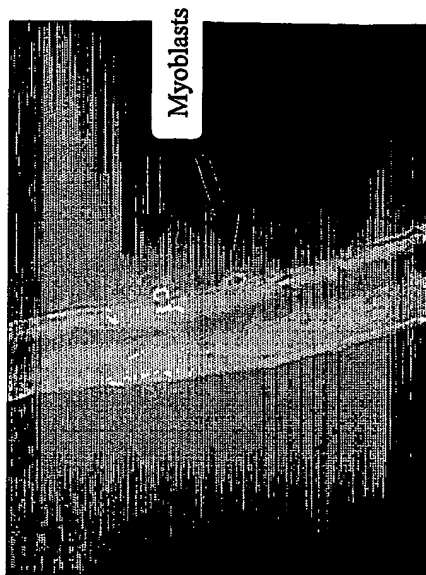
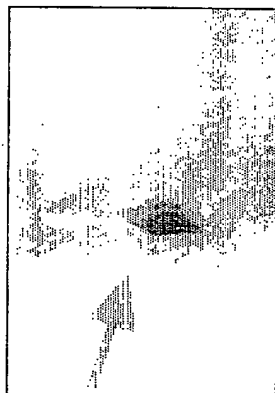


FIG.12

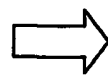
Isolation and culture of myoblast



Harvested Muscle



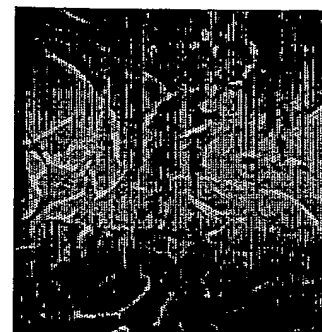
Collagenase 37°C, 30min



Desmin stained Myoblasts



Myoblasts in culture



# Methods: Myoblast Sheet Construction

FIG.13

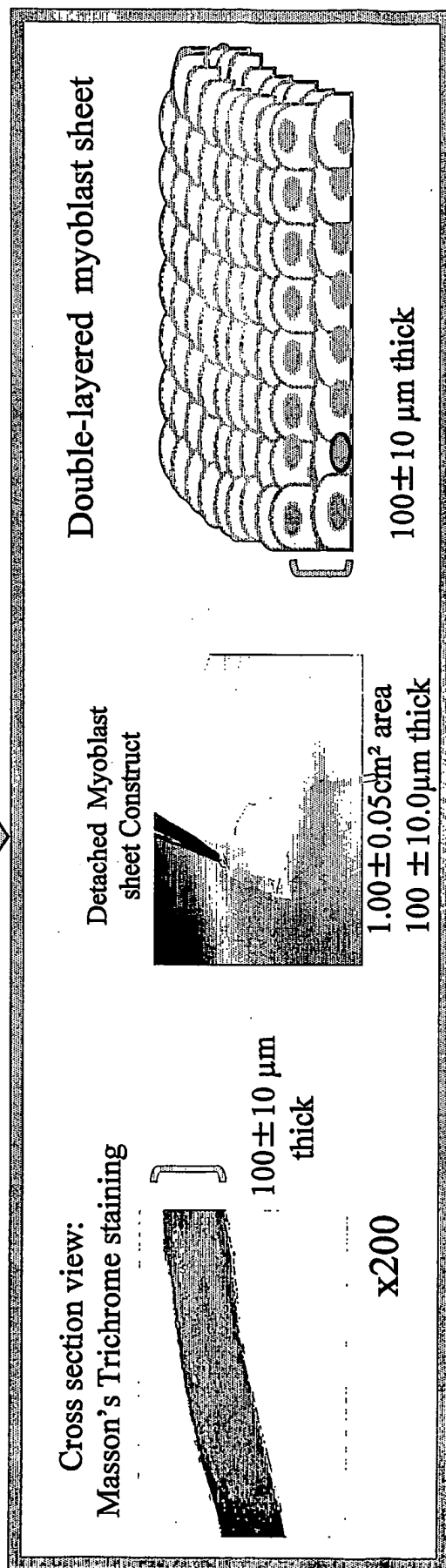
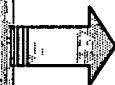
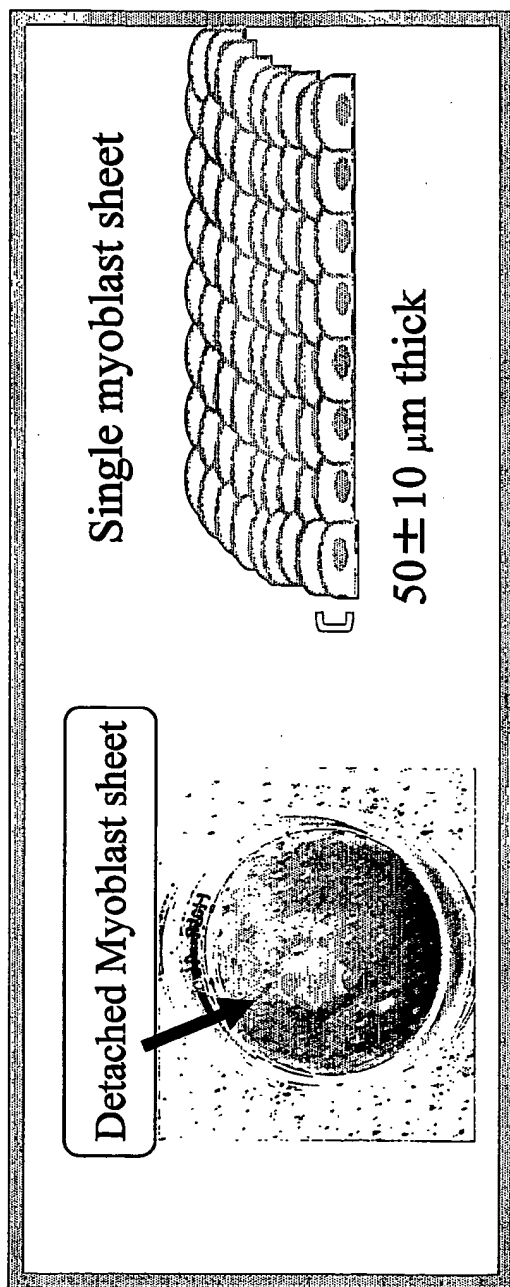
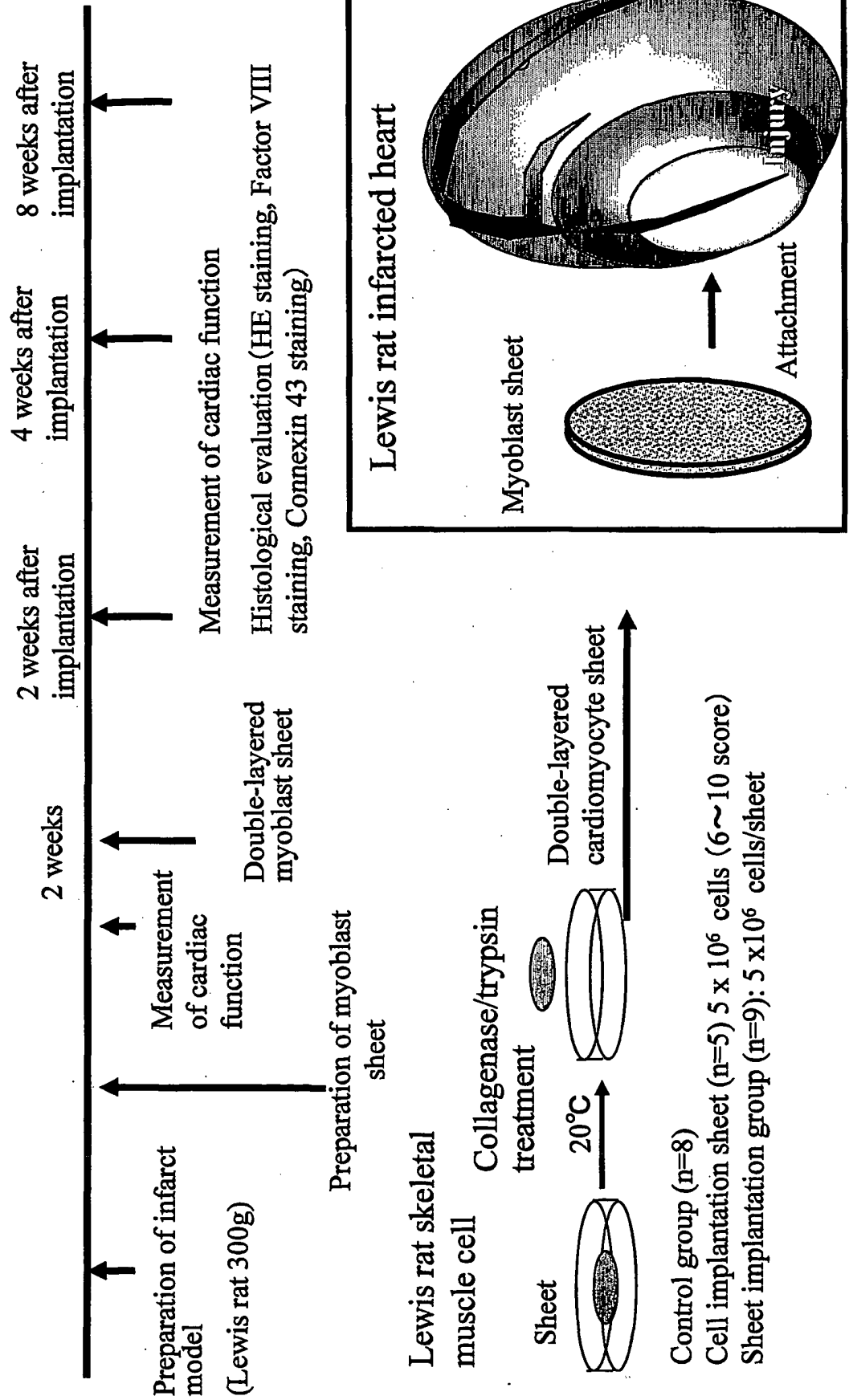


FIG.14

# Experimental Protocol



**FIG.15**

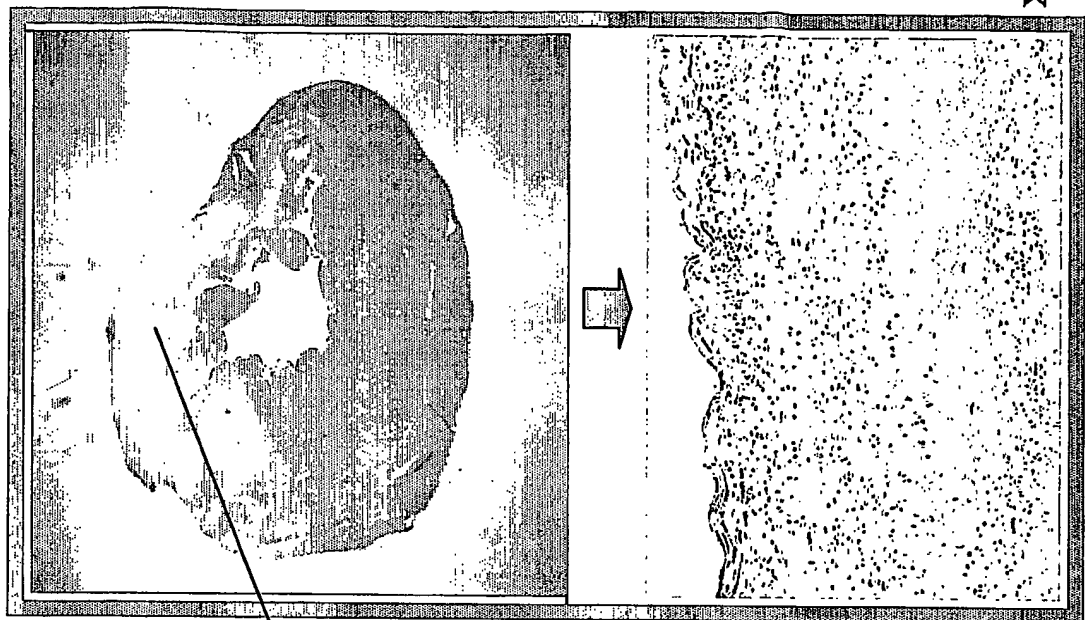
Myoblast sheet: 4W post implantation

Implanted myoblasts



x1000

HE staining

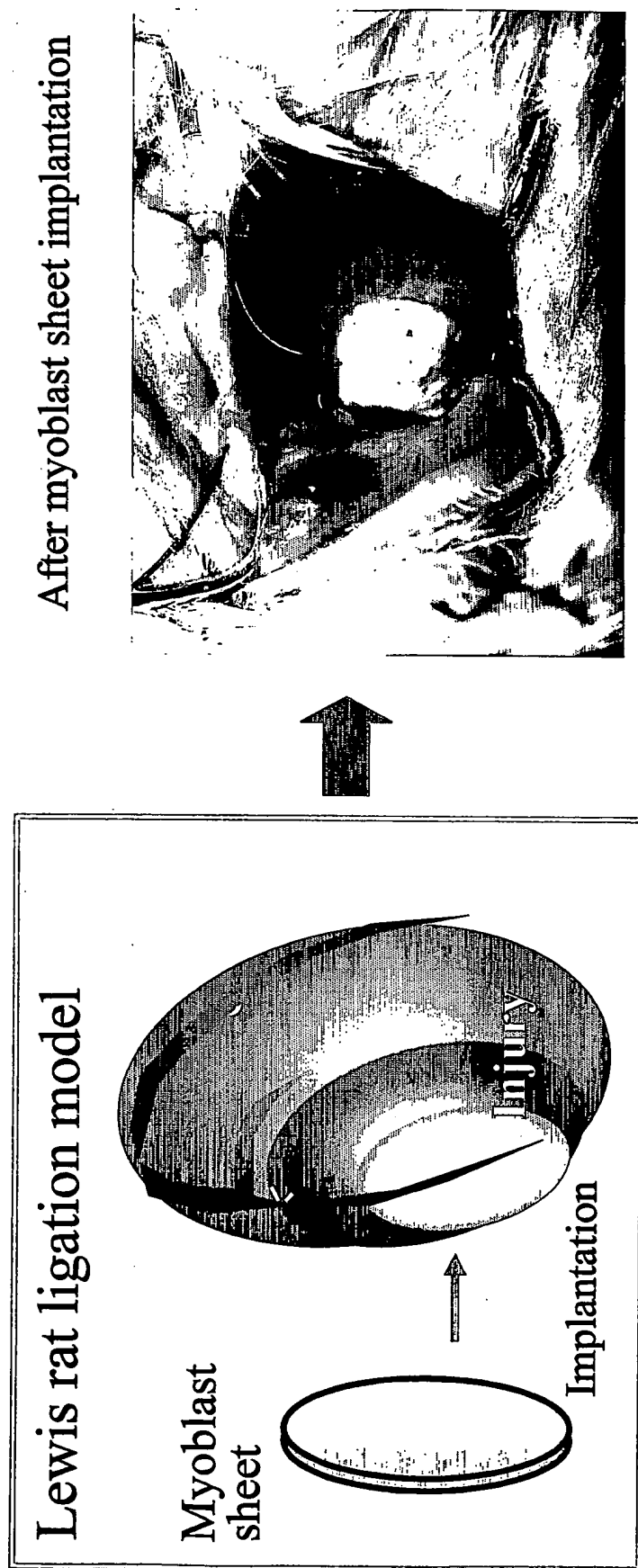


x10

x200

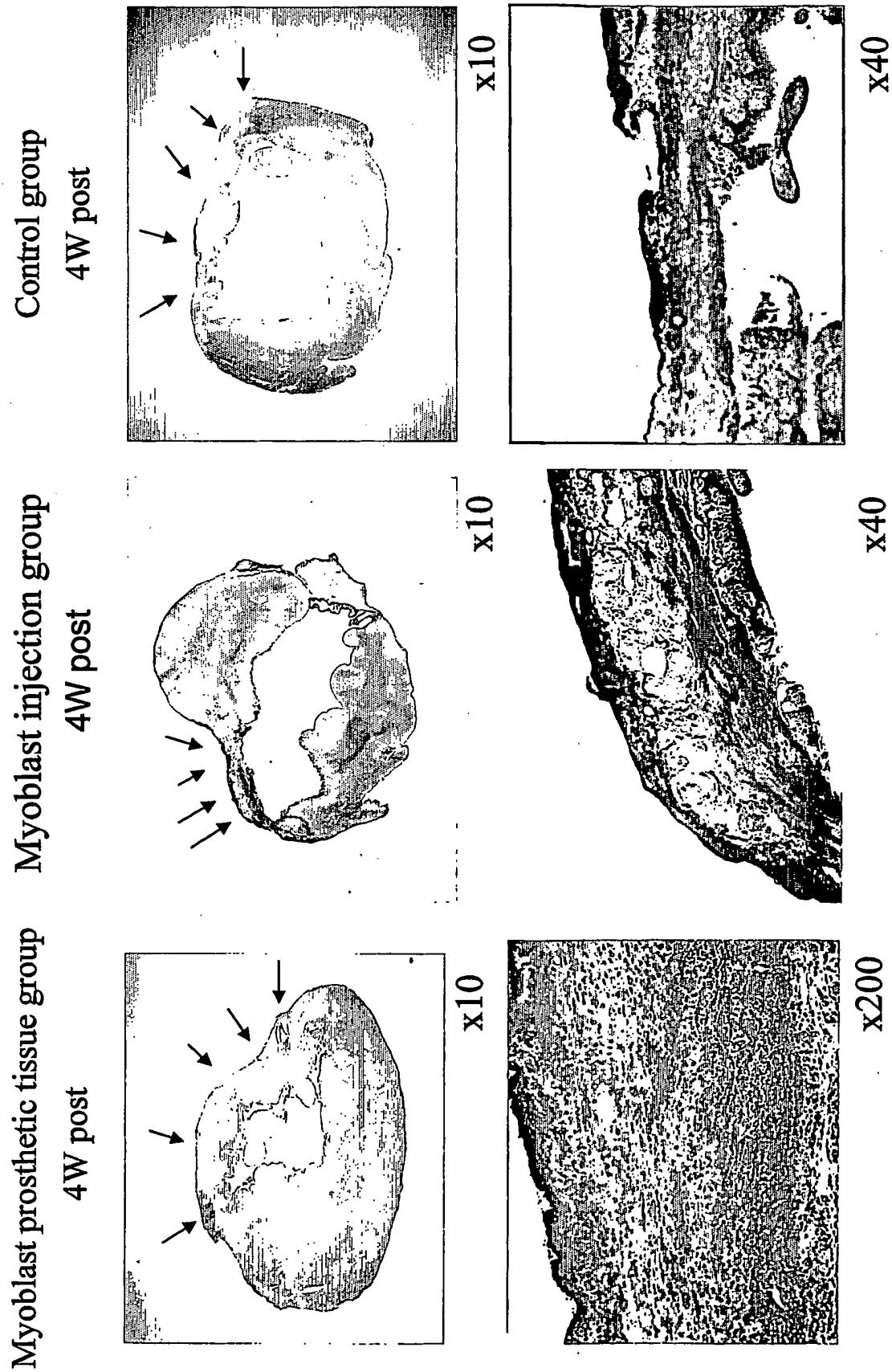
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**FIG.16**      Myoblast sheet Implantation procedure



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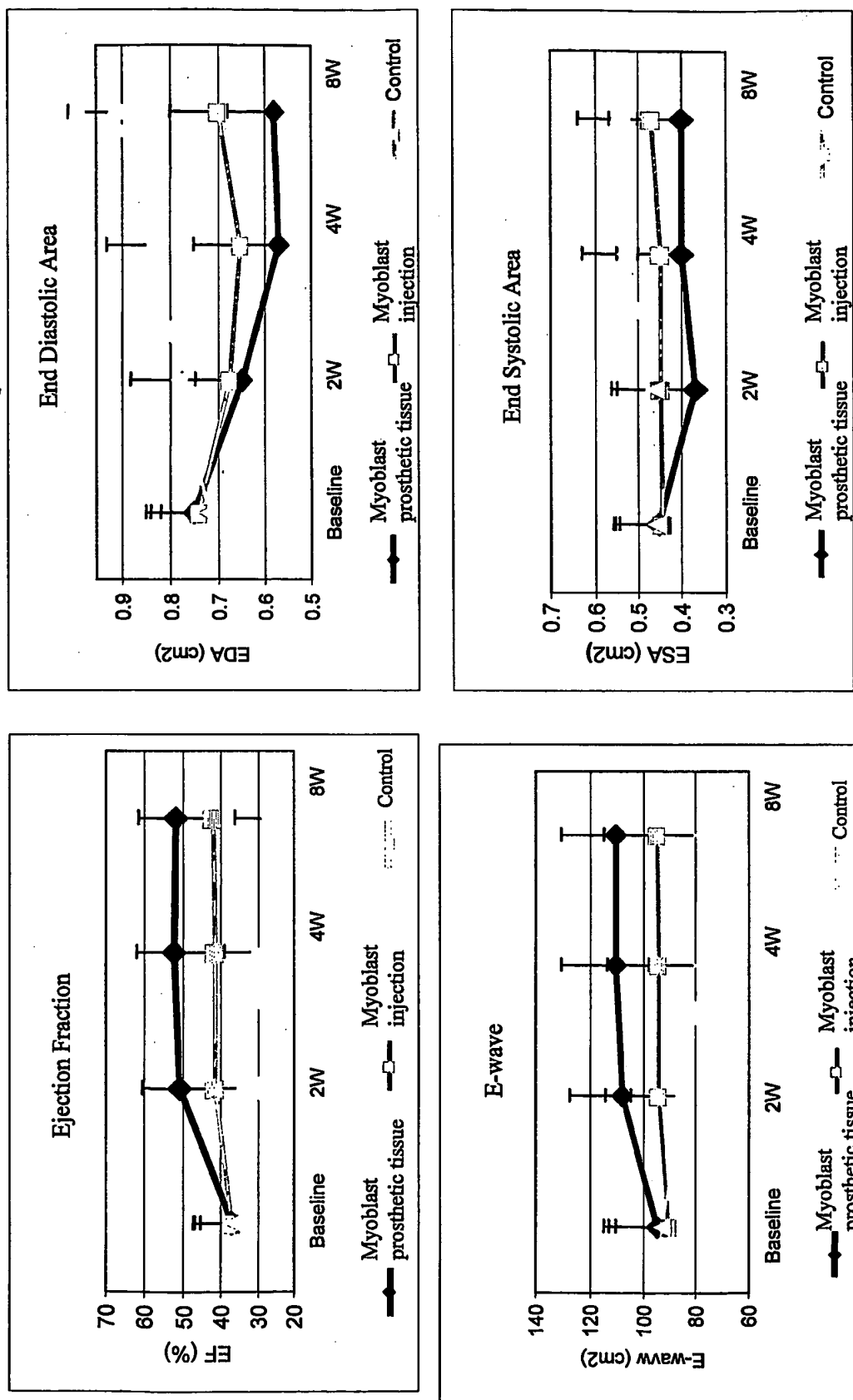
# **FIG.17**      HistologyMasson's Trichrome staining





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FIG.19



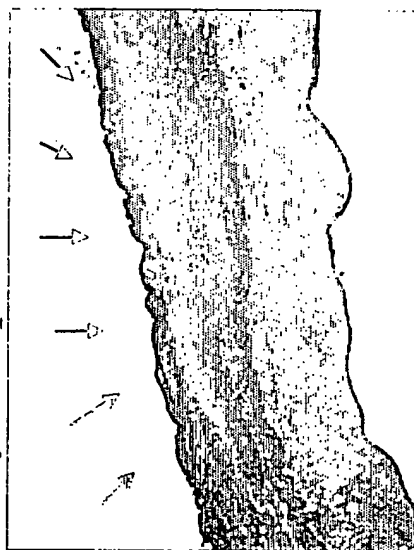
#P< 0.05 for control; \*P< 0.05 to for injection needle group



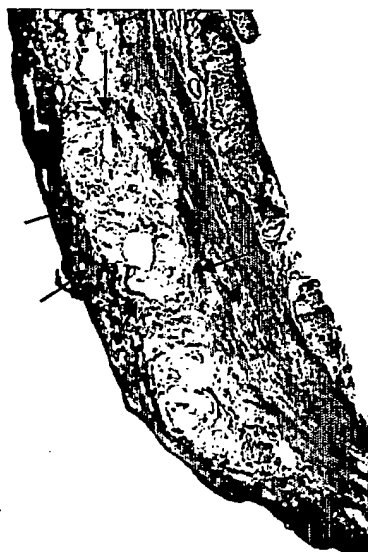
FIG.20

# Anterior Wall Thickness Comparison

Myoblast prosthetic tissue



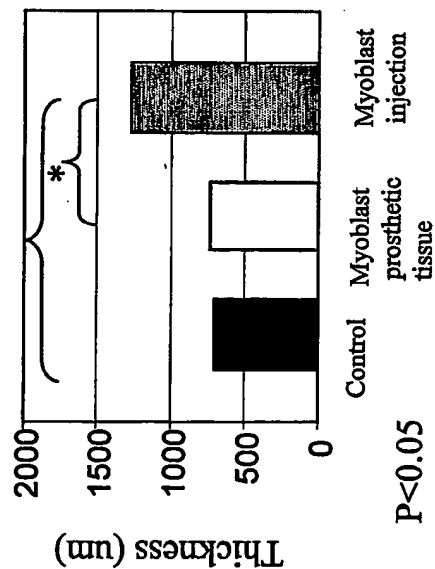
Myoblast injection



x40

x40

Anterior wall thickness



Control



x40

**FIG.21**

Myoblast sheet:  
Desmin Staining



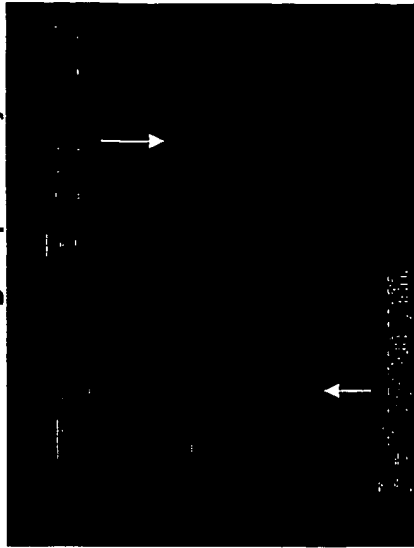
x100

Control group (GFP)



x100

Myoblast prosthetic  
tissue group (GFP)



x100  
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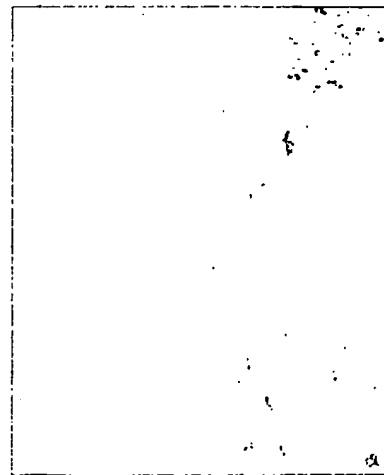
Factor VIII staining

Myoblast injection



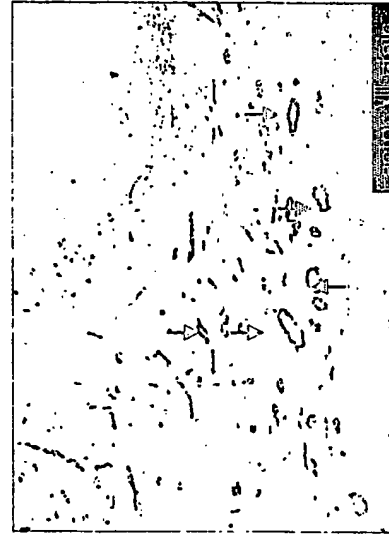
x40

Control



x40

Myoblast prosthetic tissue



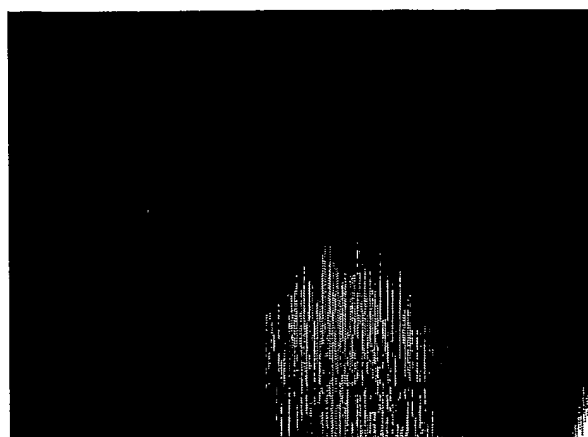
x40

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**FIG.22A**



**FIG.22B**



**FIG.22C**



FIG.22D

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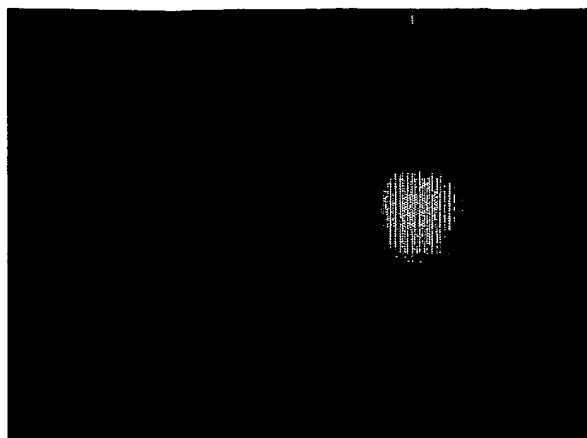
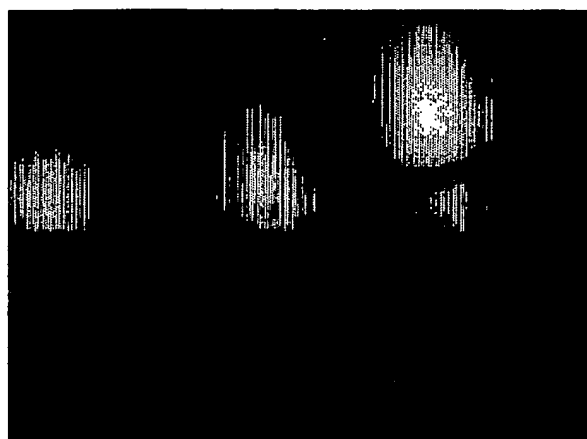


FIG.22E



FIG.22F



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FIG.23A



FIG.23B

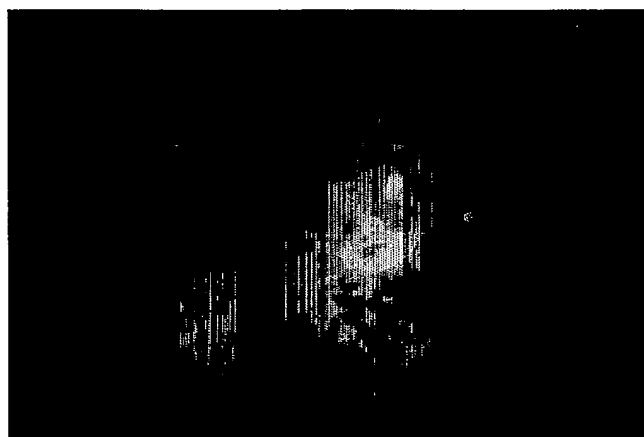
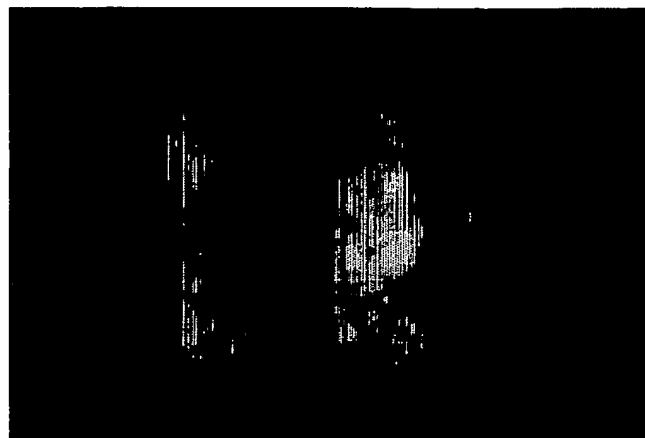
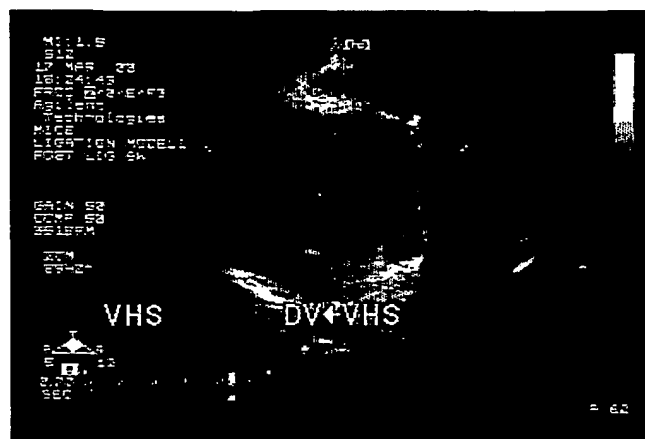


FIG.23C



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FIG.24A



**FIG.24B**

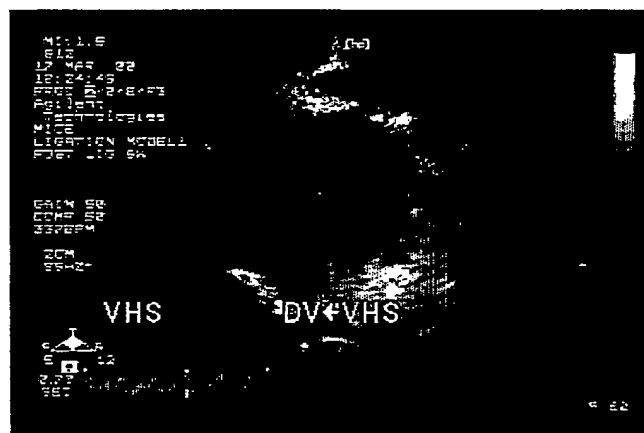
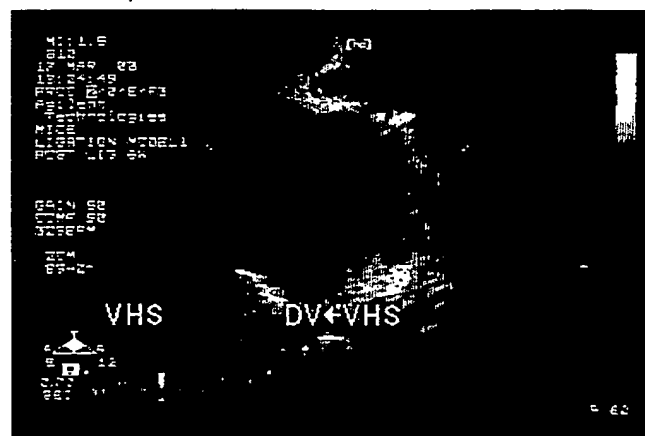


FIG.24C



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FIG.25A

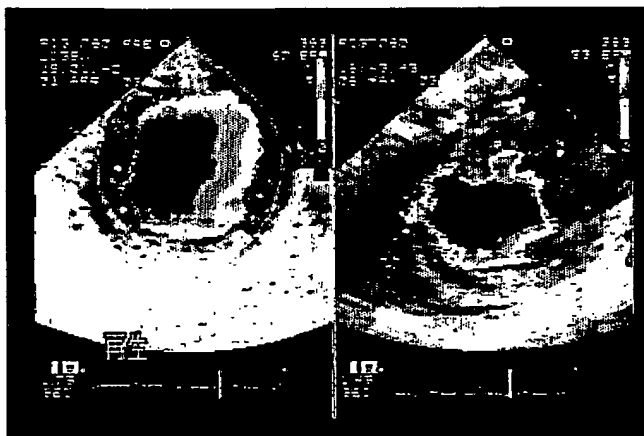


FIG.25B

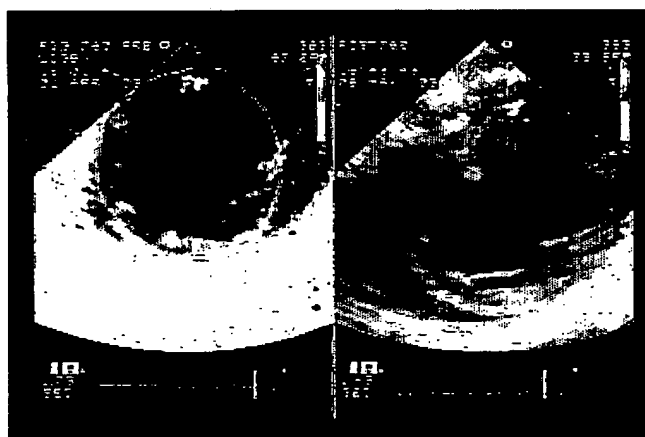
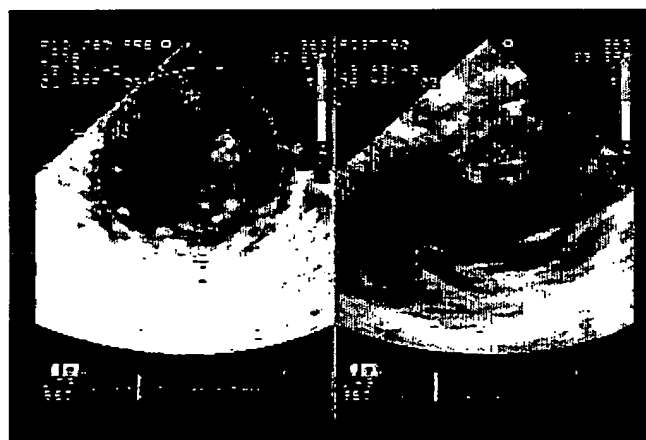


FIG.25C



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FIG.26A

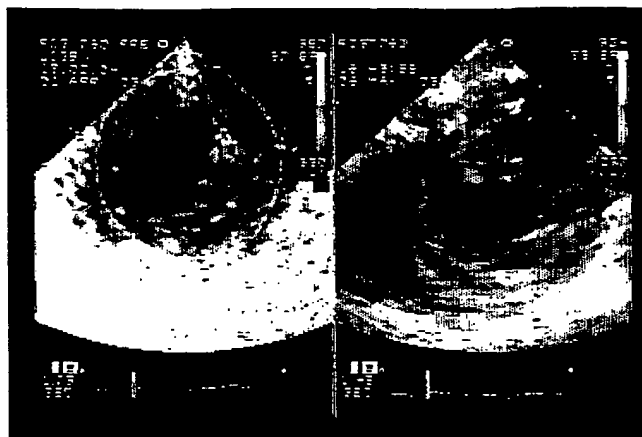


FIG.26B

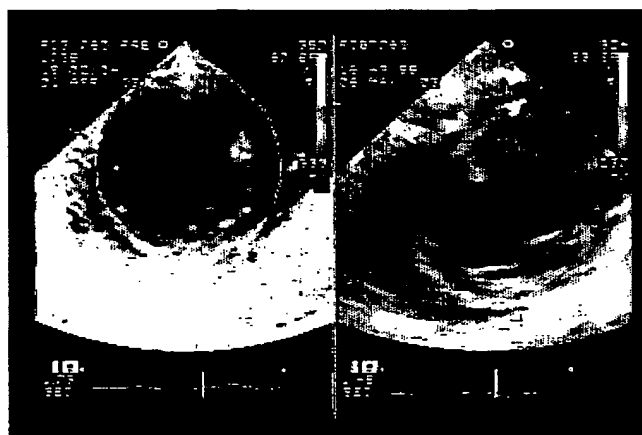


FIG.26C





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FIG.27A

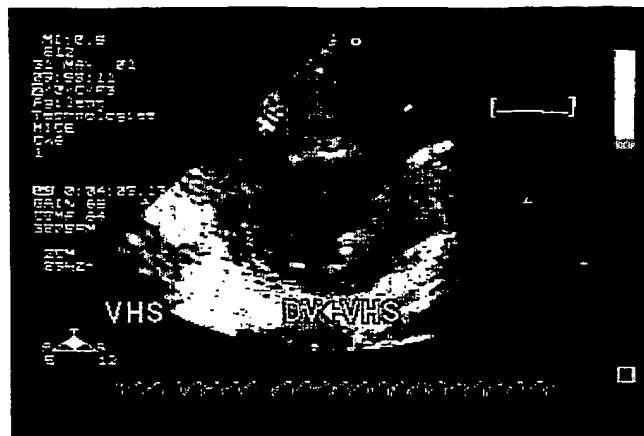


FIG.27B

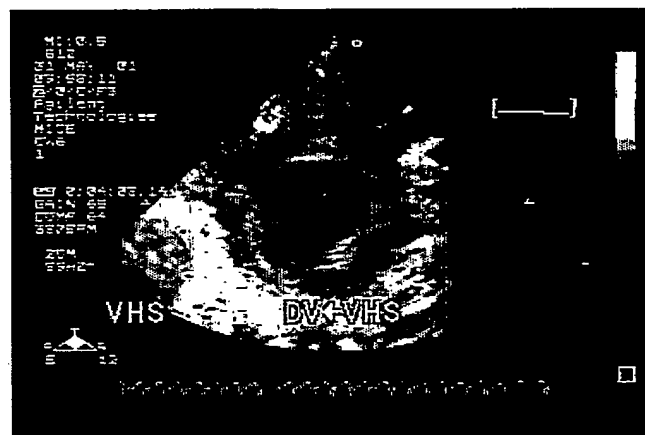
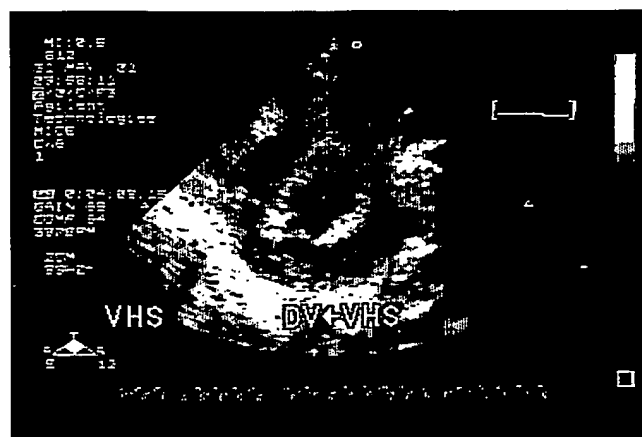


FIG.27C



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FIG.28

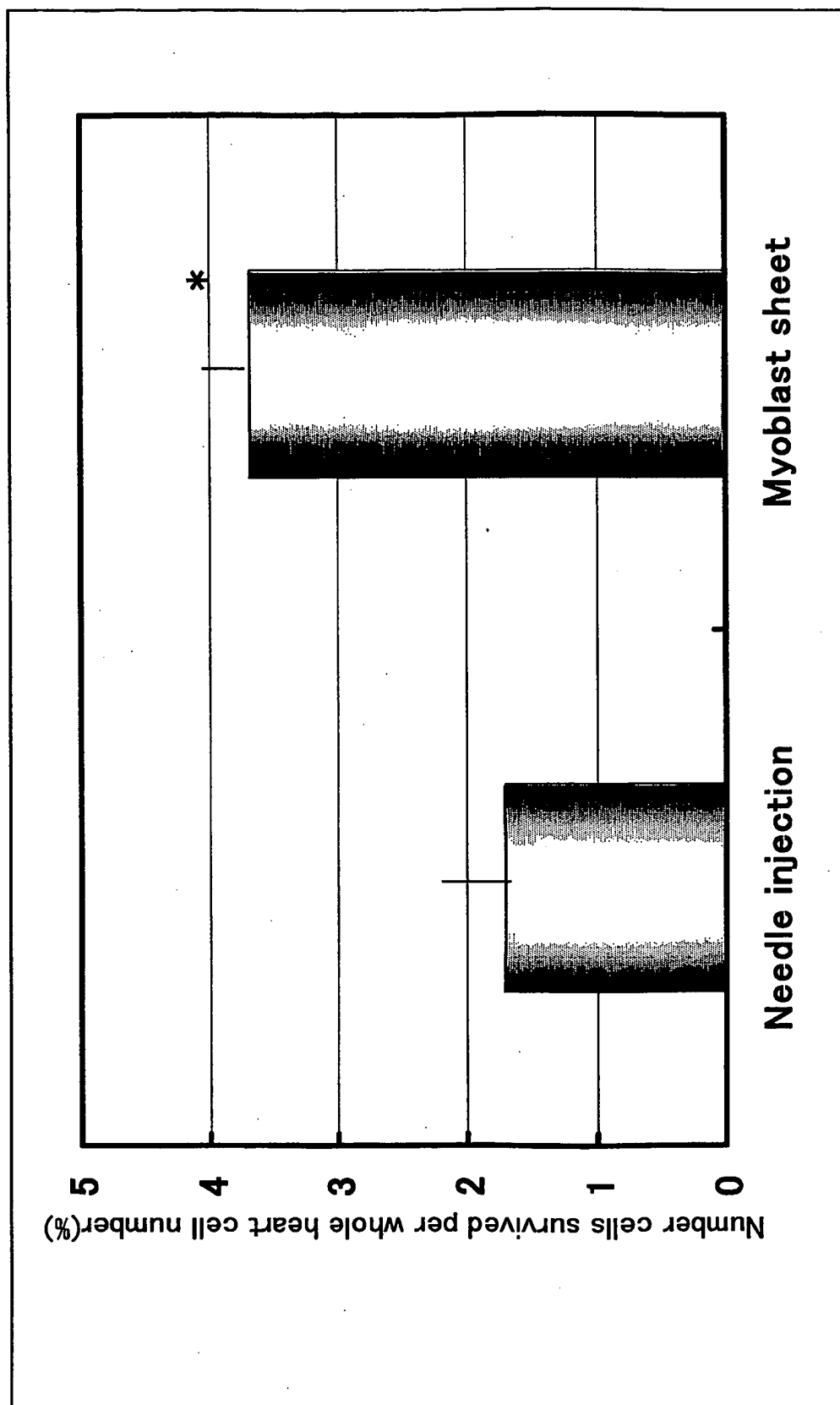


FIG.29

Masson's Trichrome staining x400

HE staining x400



MHC fast x400

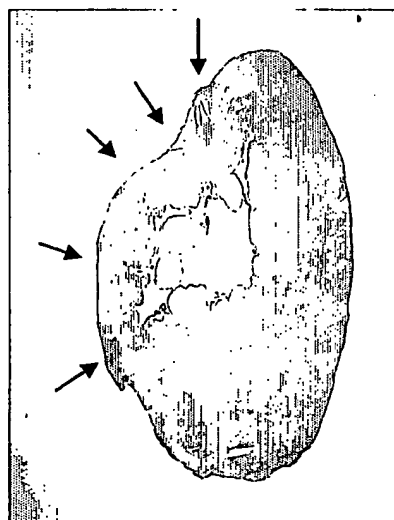
MHC slow x400

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# FIG.30A Tissue (Masson's Trichrome staining)

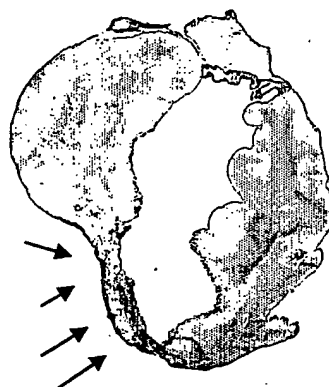
Myoblast sheet  
4W post implantation

x10



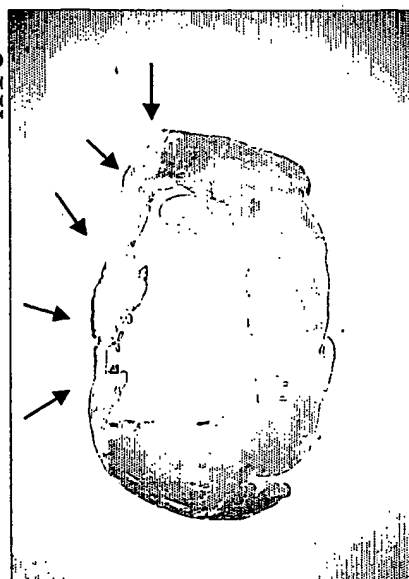
Myoblast implantation  
(needle injection)

4W post implantation x10



Control

x10



x40



x40

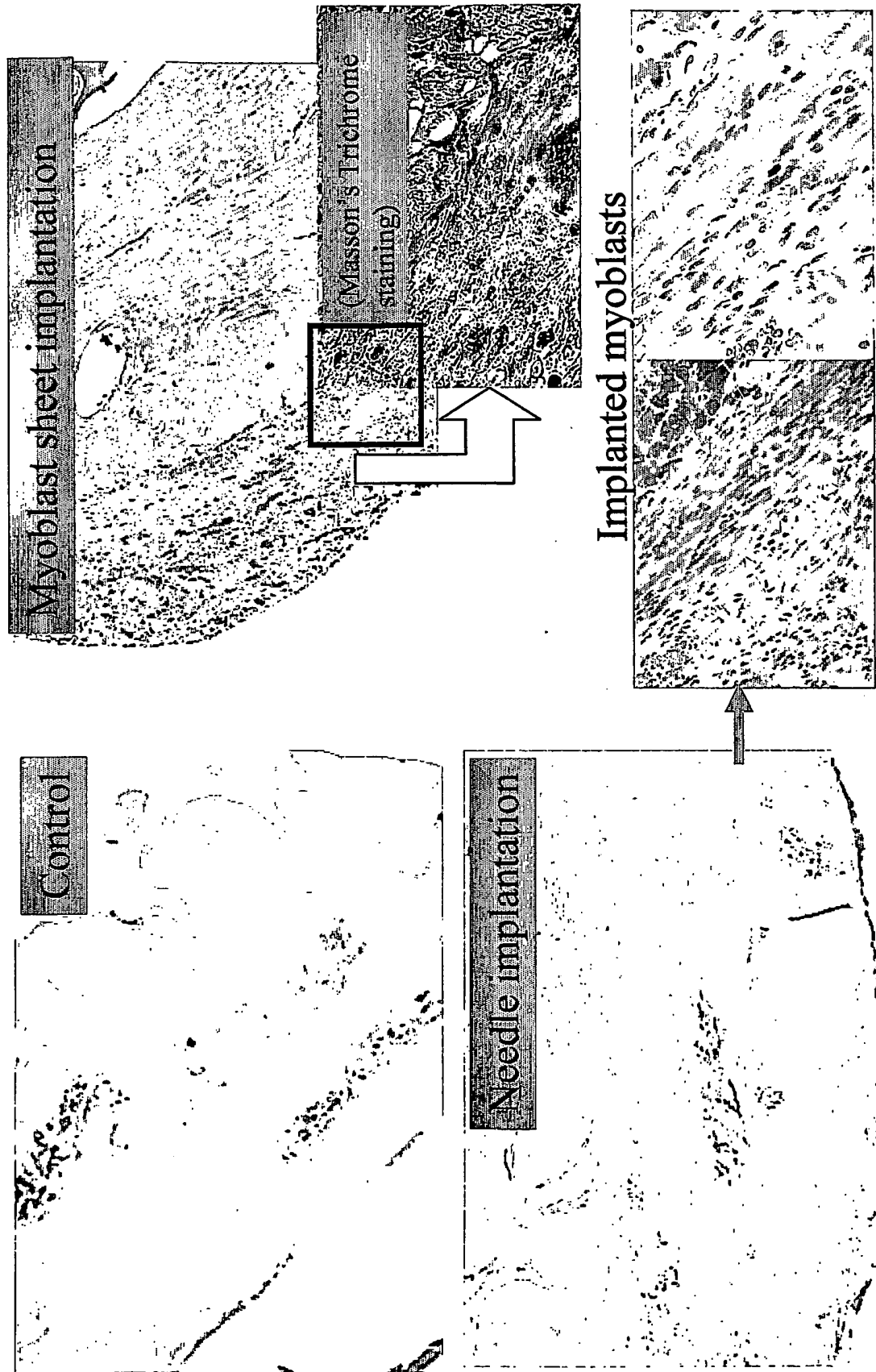


x40



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FIG.30B



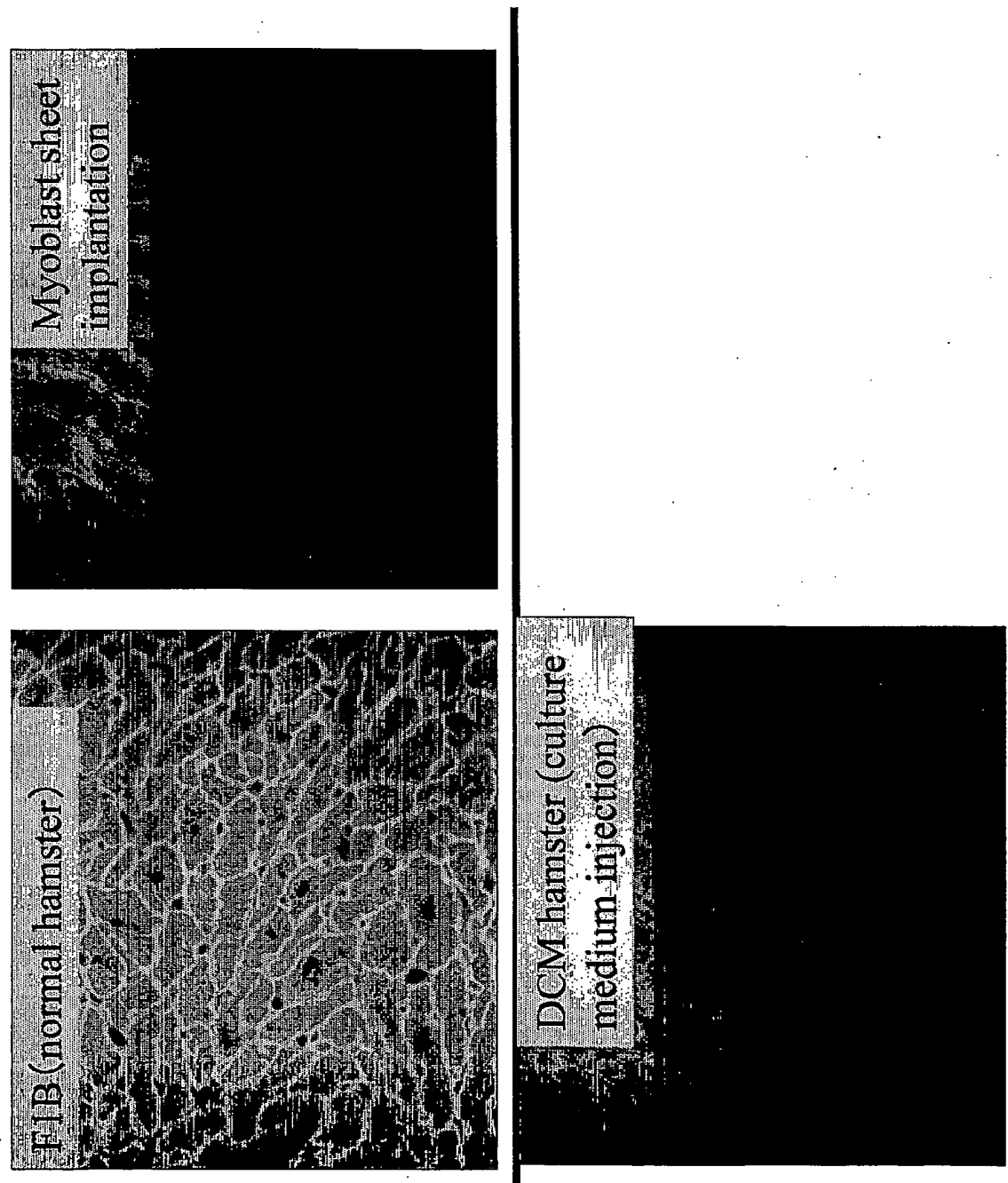


FIG.30C

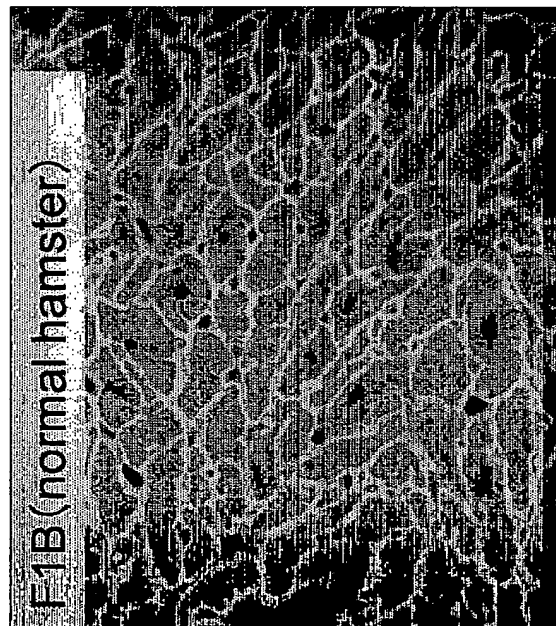


FIG.30D

FIG.31 Survival rate of implanted cell

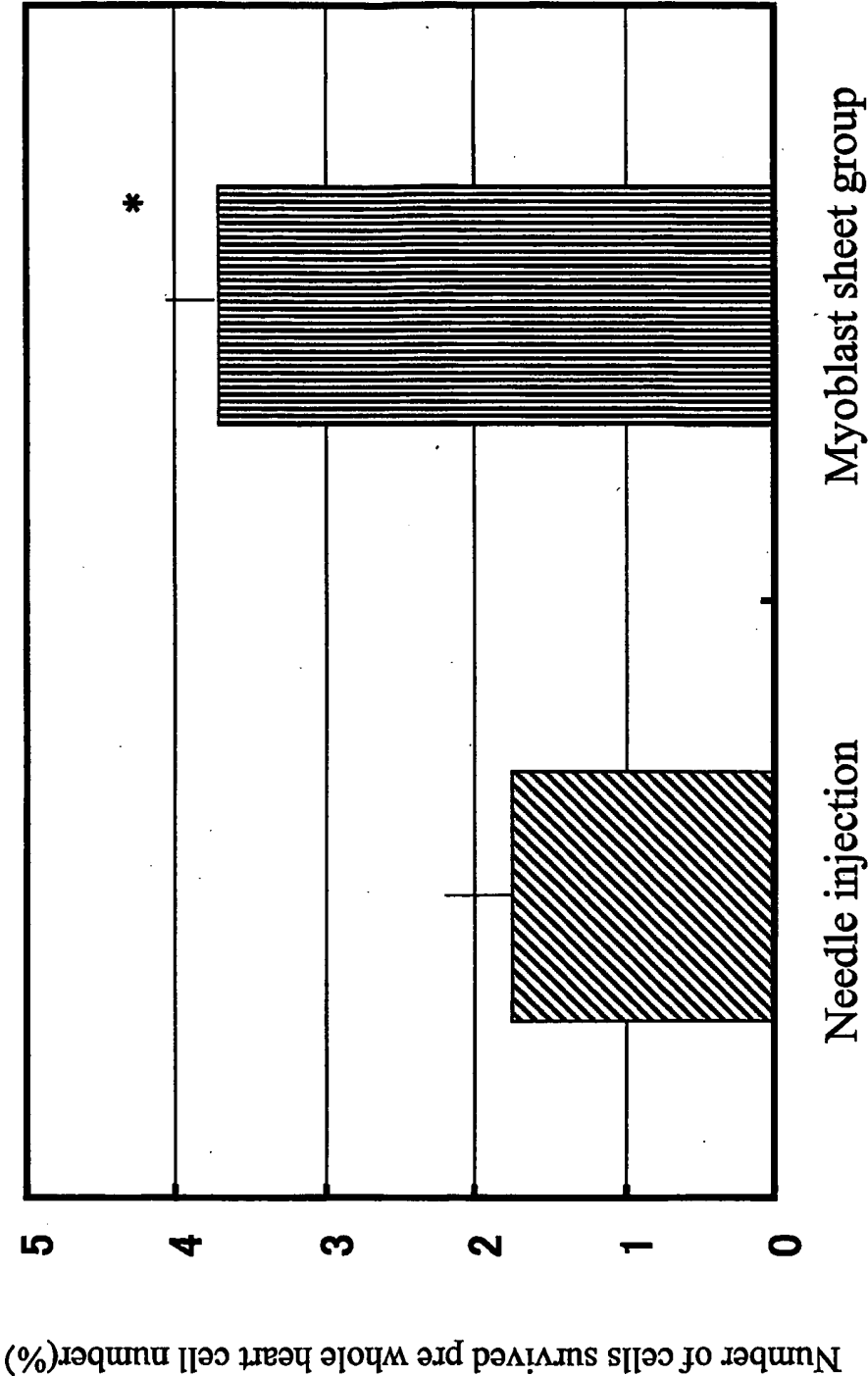


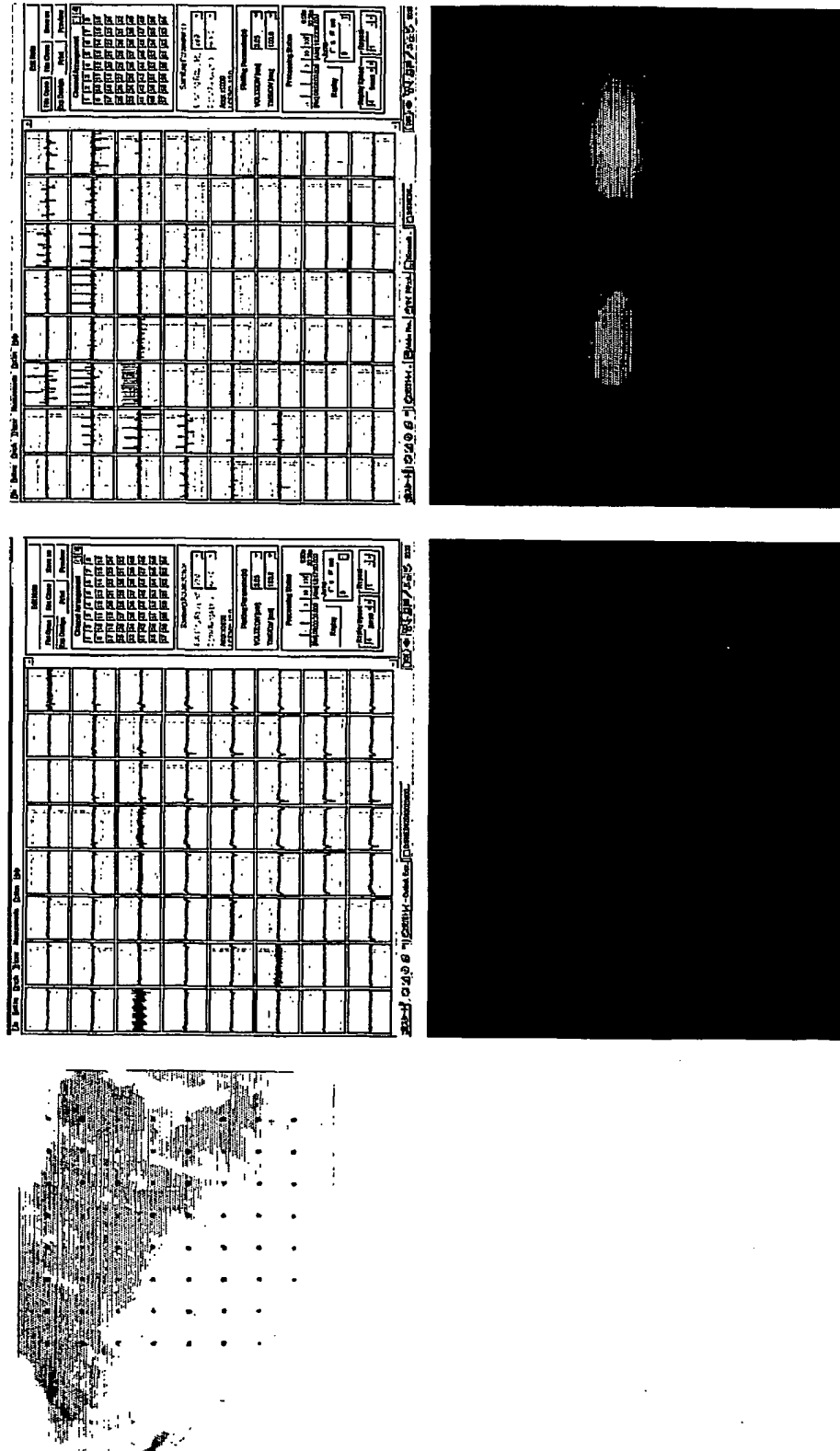


FIG.32  
MED system

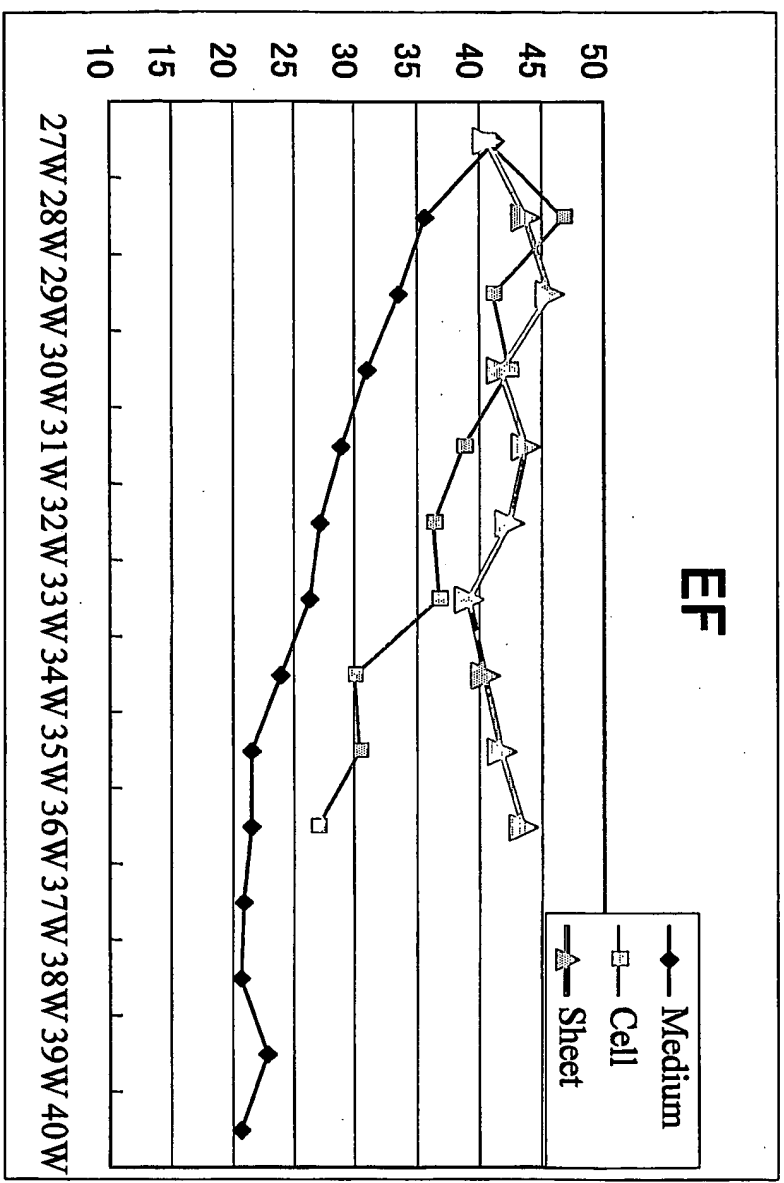
Electronical properties of myoblast sheet

Cardiomyocyte sheet

Myoblast sheet



**FIG.33A** Myoblast sheet implantation to dilated  
cardiomyopathic hamster



HE staining



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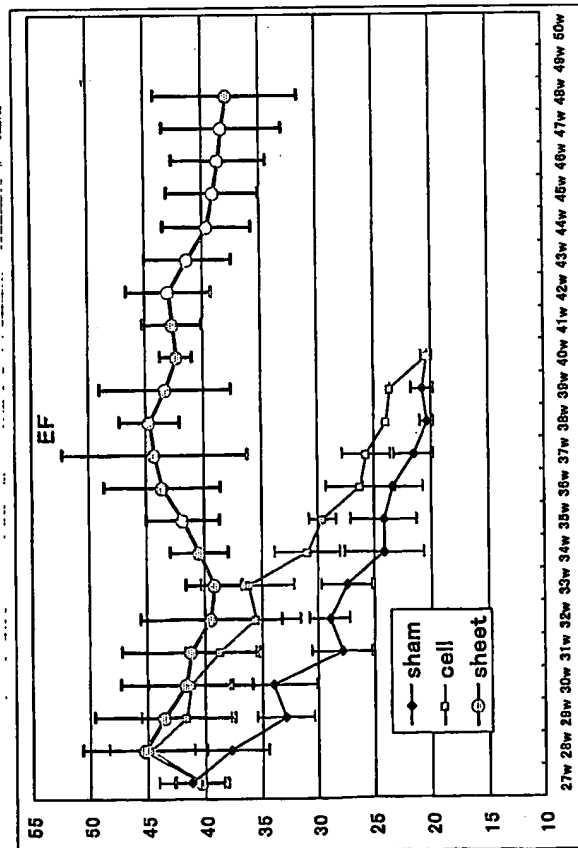
Masson's Trichrome staining



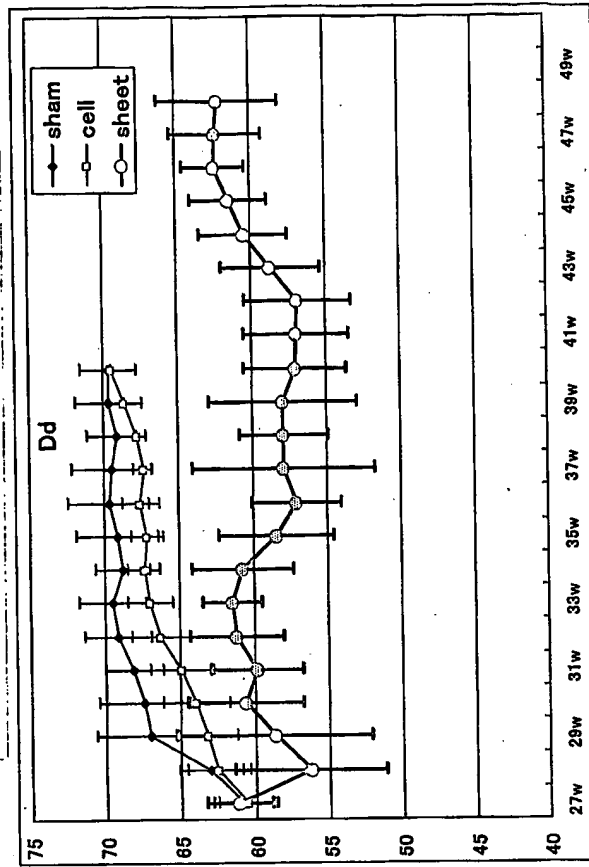
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FIG.33B

Left ventricular end-  
systolic diameter



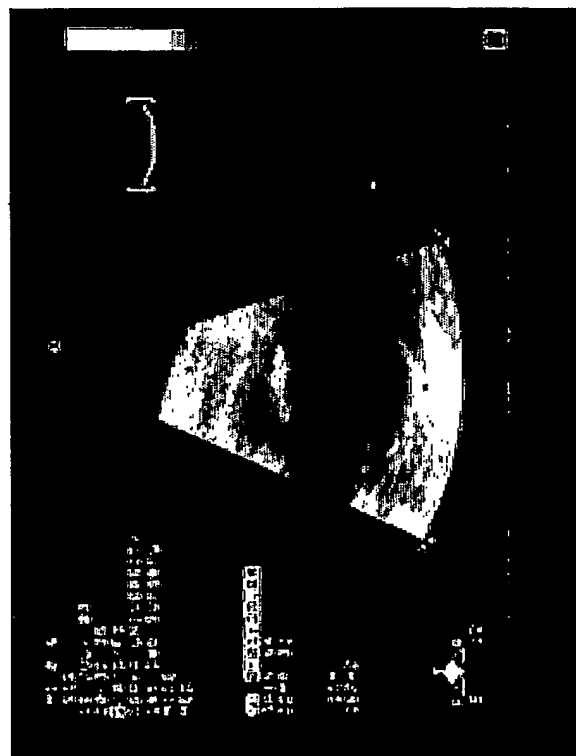
Left ventricular end-  
diastolic diameter



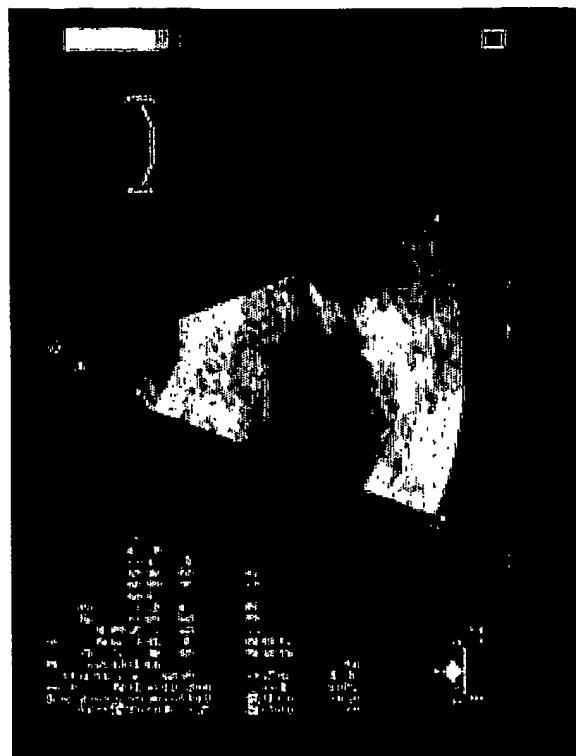
40 /47

FIG.33C

Control group

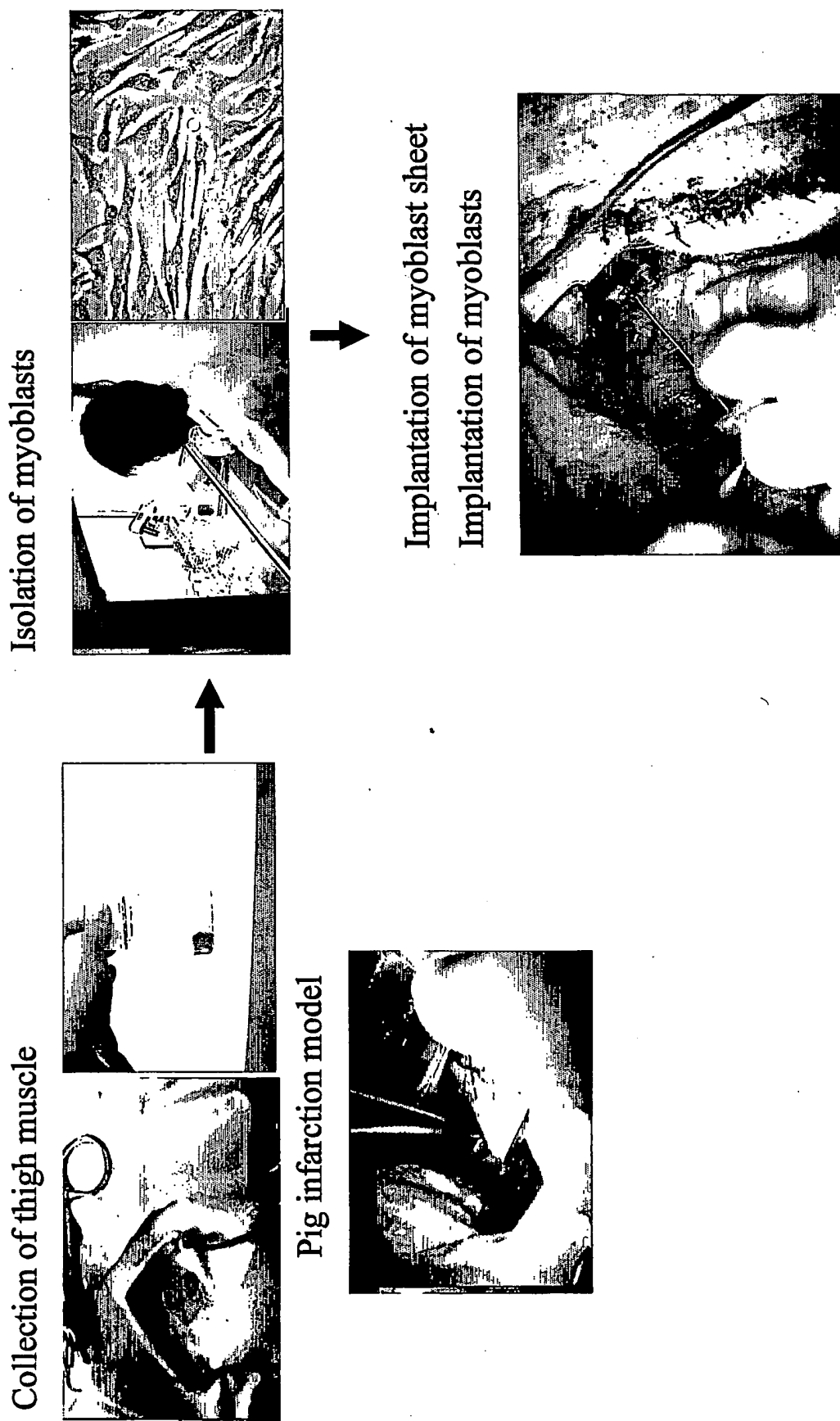


Myoblast sheet implantation group



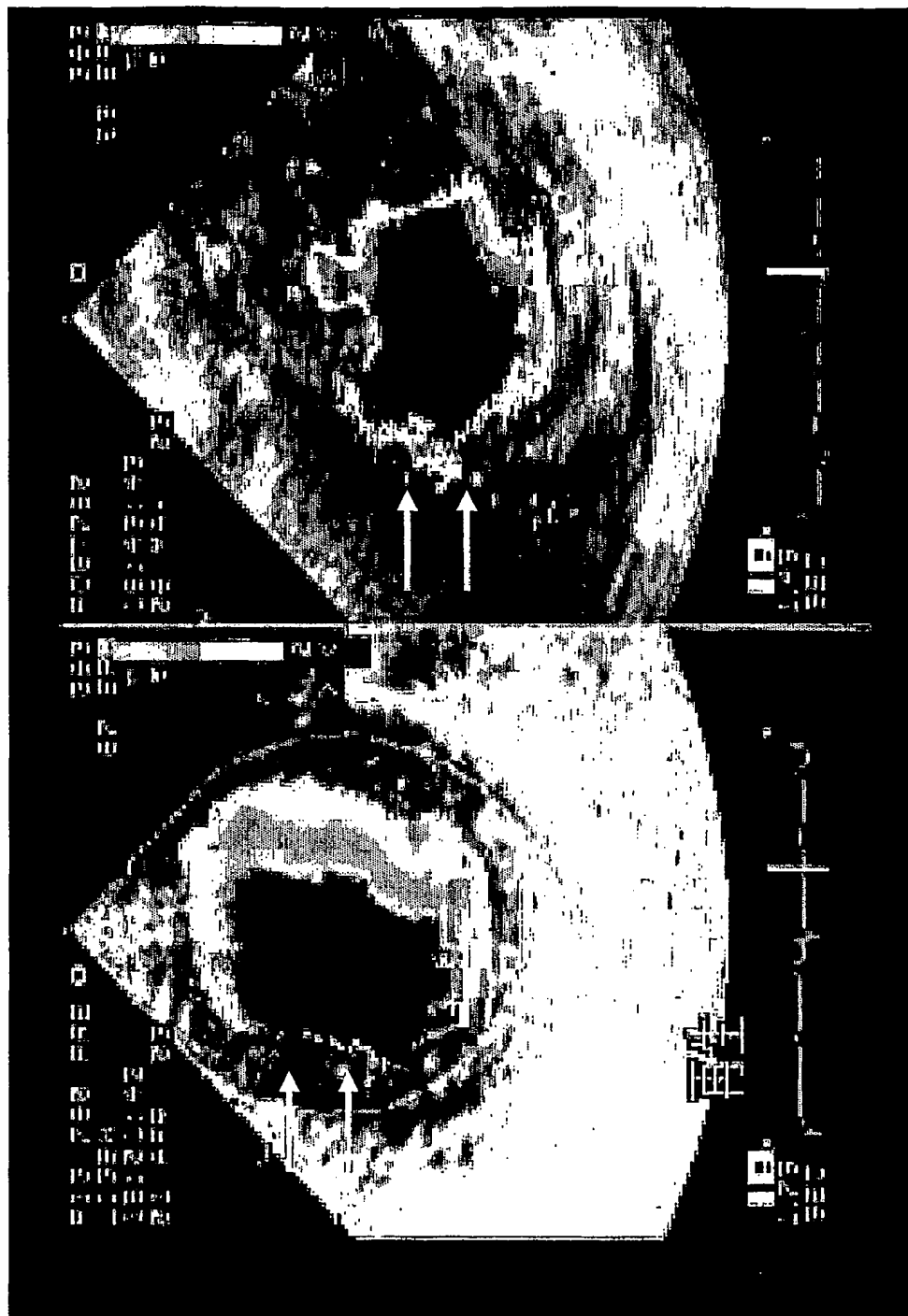
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# FIG.34      Myoblast sheet implantation into pig infarction model



**FIG.35**  
Evaluation of cardiac function (systolic function) of  
pig infarction model by CKI method

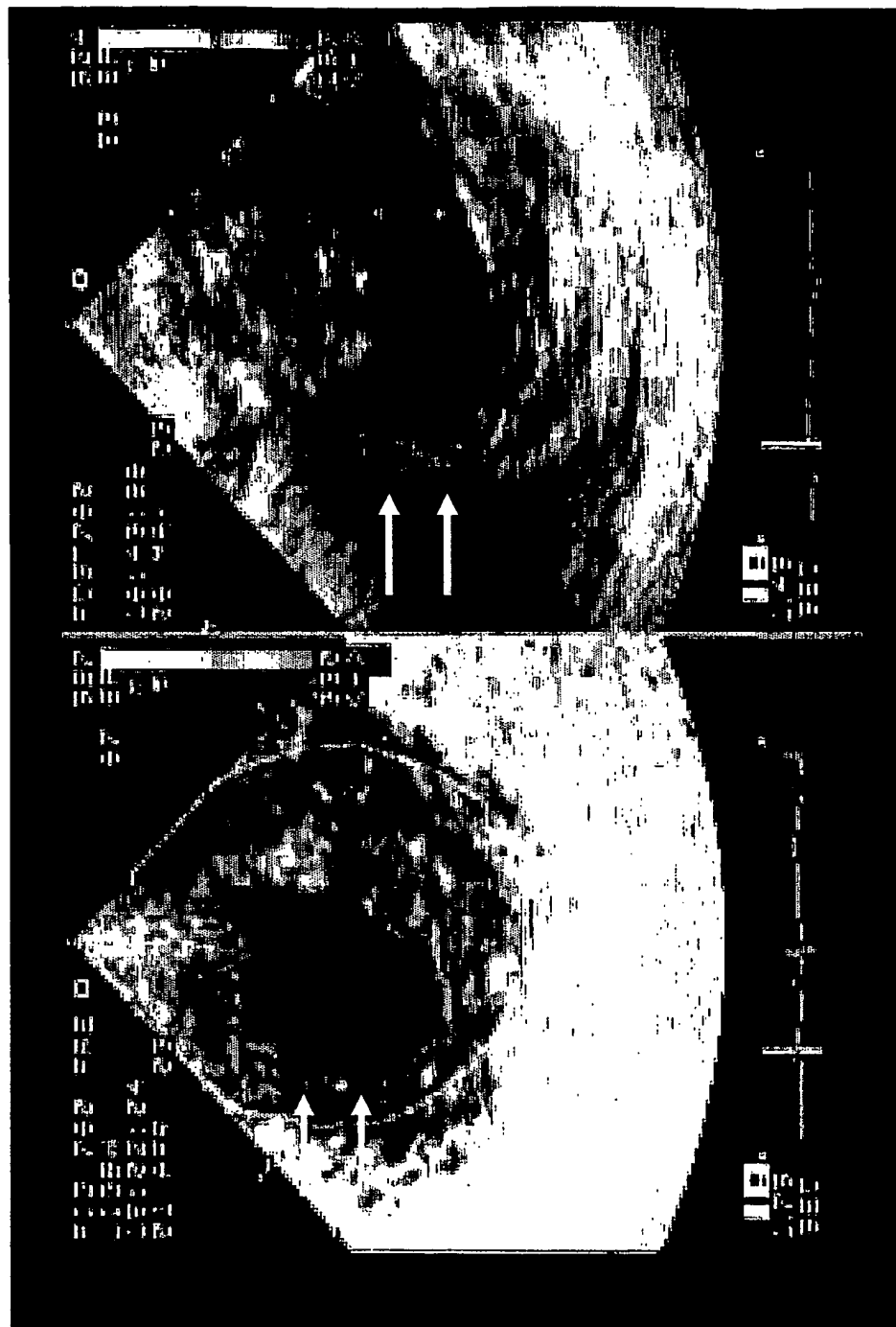
Before operation      After operation



**FIG.36**

Evaluation of cardiac function (diastolic function) of  
pig infarction model by CKI method

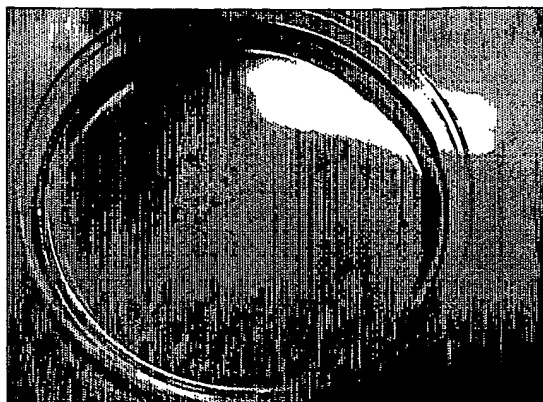
Before operation                      After operation



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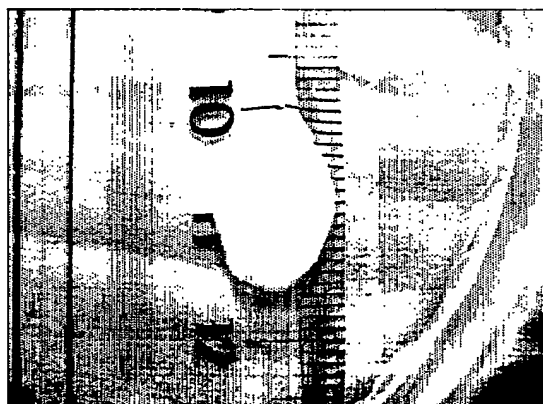
**FIG.37**

Without ascorbic acid

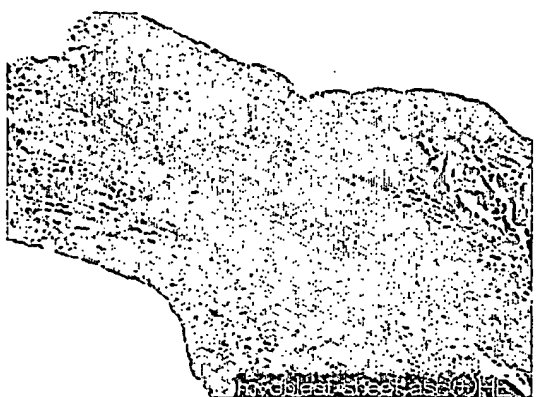


**FIG.38**

With ascorbic acid



**FIG.39**





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FIG.40

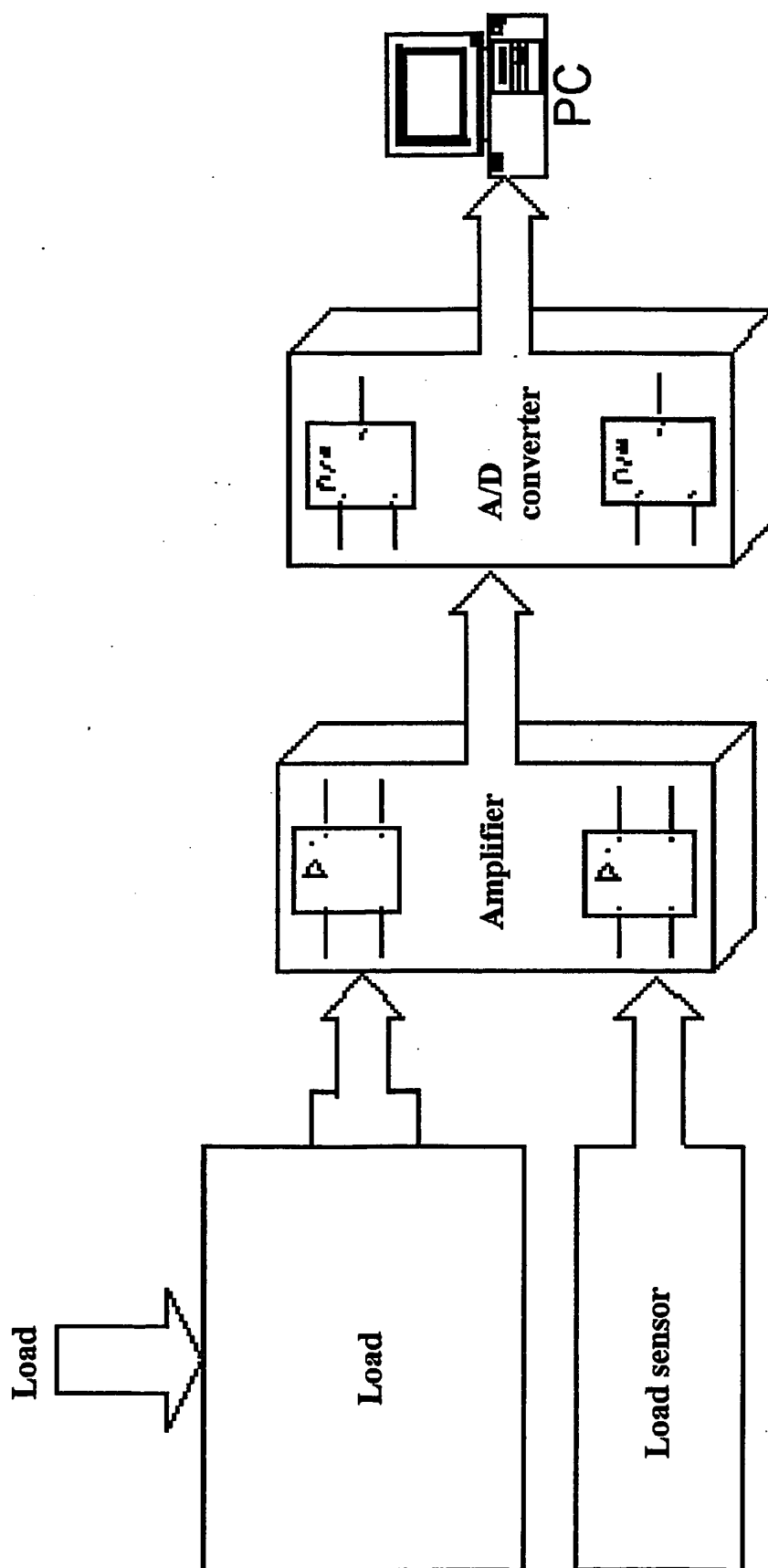
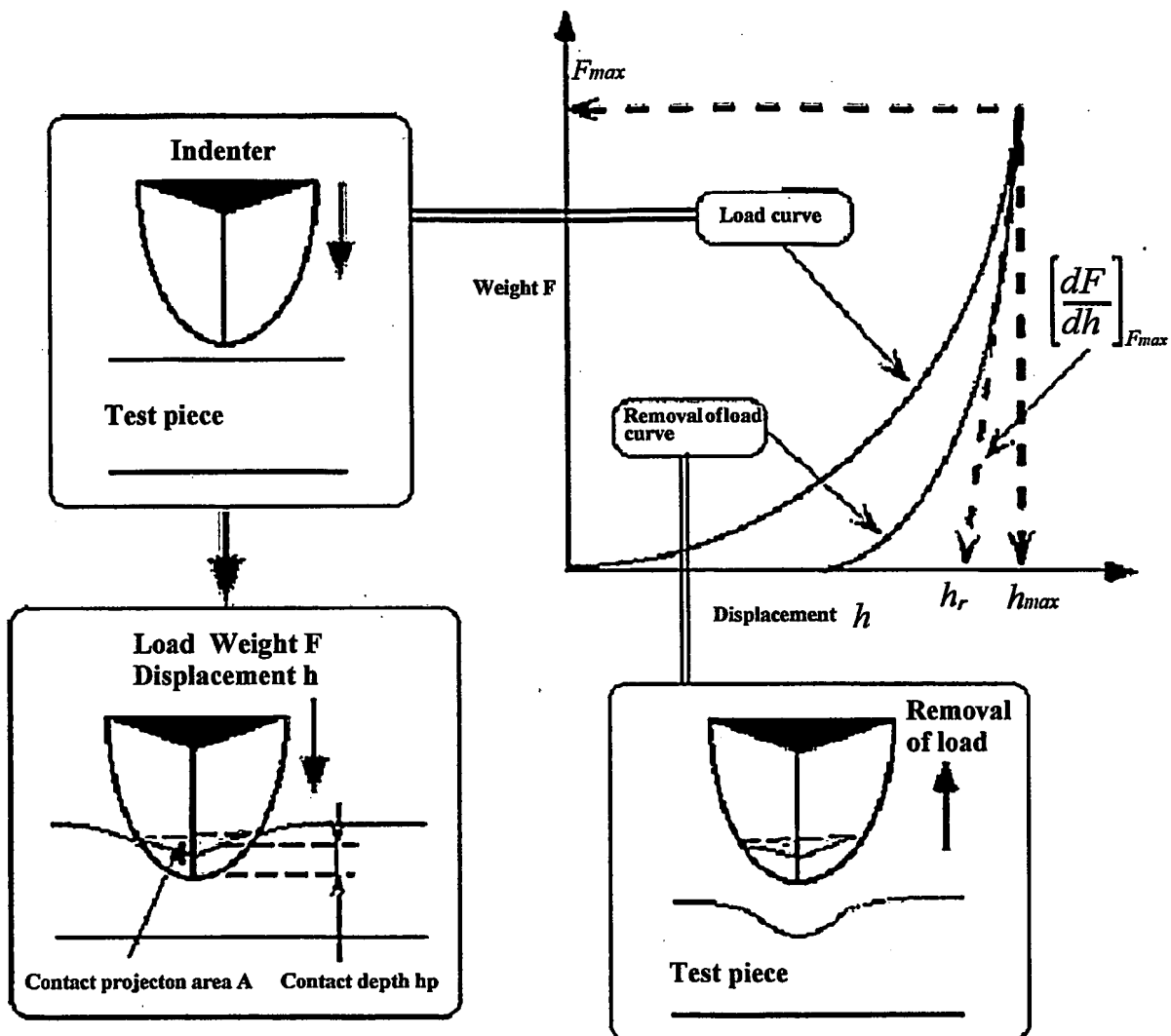


FIG.41



Rigidity  $H = \frac{F}{A} = \frac{F}{k_1 h_p^2}$

Young's modulus  $E = \left[\frac{dF}{dh}\right]_{F_{max}} \frac{1 - \nu^2}{2 \cdot k_2 \cdot h_{pmax}}$

Contact depth  $h_p = h_r + 0.25(h_{max} - h_r)$

$F$ : Load

$A$ : Contact projection area

$h_p$ : Contact depth

$k_1 k_2$ : Shape coefficient

$F_{max}$ : Maximum load

$h_{max}$ : Max. displacement

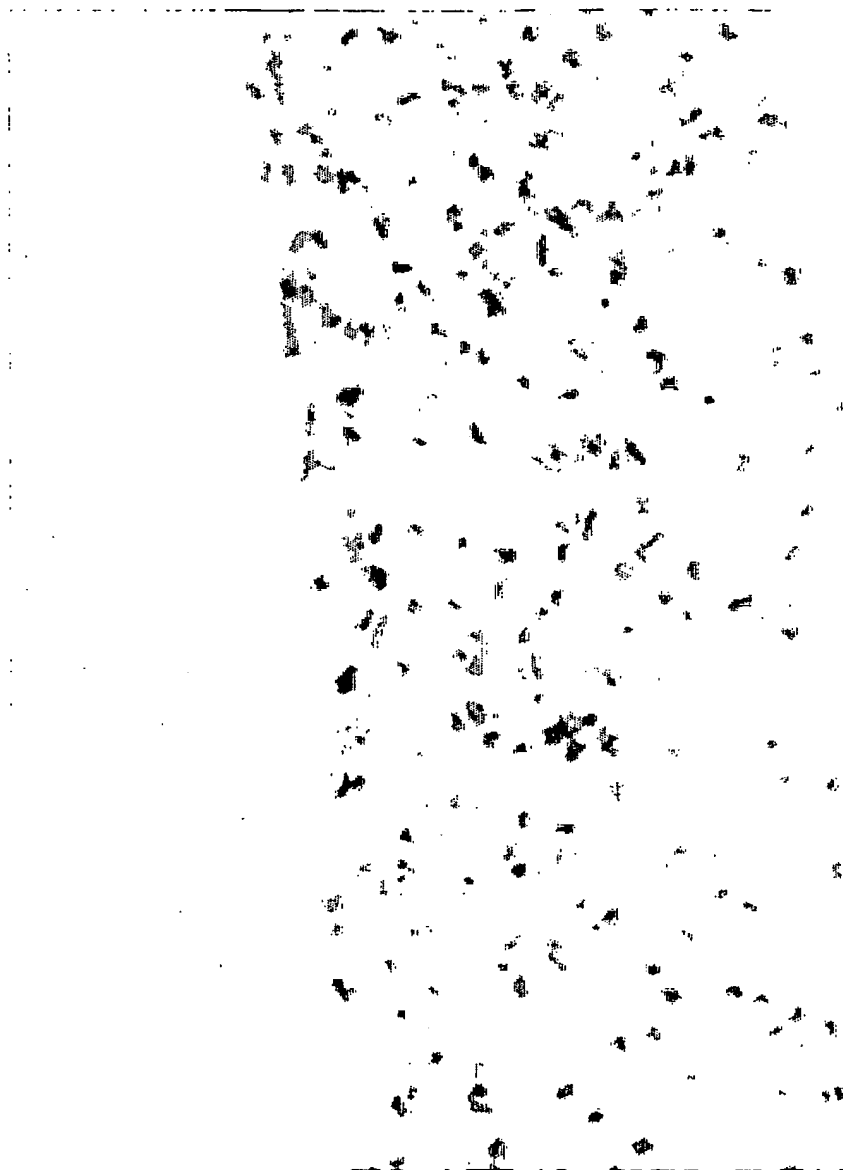
$h_r$ : Point at which tangential line cross weight 0

$dF/dh$ : Gradient of tangential line of the removal of load curve

$\nu$ : Poisson's ratio

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FIG.42



**10/567728**

## SEQUENCE LISTING

**IAP5 Rec'd PCT/PTO 31 JAN 2006**

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 5 <120> three dimensional tissue construct  
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## PCT REQUEST

Original (for SUBMISSION)

VIII-5-1	<b>Declaration: Non-prejudicial disclosure or exceptions to lack of novelty</b> Declaration as to non-prejudicial disclosures or exceptions to lack of novelty (Rules 4.17(v) and 51bis.1(a)(v)): Name (LAST, First)	<b>in relation to this international application</b>  <b>Cardio Incorporated declares that the subject matter claimed in this international application was disclosed as follows:</b>
VIII-5-1(i)	Kind of disclosure:	<b>other: Scientific Meeting</b>
VIII-5-1(ii)	Date of disclosure:	<b>28 March 2003 (28.03.2003)</b>
VIII-5-1(iii)	Title of disclosure:	<b>The 67th Annual Scientific Meeting of the Japanese Circulation Society</b>
VIII-5-1(iv)	Place of disclosure:	<b>Room 10</b>
VIII-5-1(i)	Kind of disclosure:	<b>other: The Website on the Internet</b>
VIII-5-1(ii)	Date of disclosure:	<b>28 March 2003 (28.03.2003)</b>
VIII-5-1(iii)	Title of disclosure:	<b>The 67th Annual Scientific Meeting of the Japanese Circulation Society</b>
VIII-5-1(v)	This declaration is made for the purposes of:	<b>all designations</b>

International Application No  
PCT/JP2004/001024

IPC 7 A61F2/00 A61L27/38 C12N5/06 A61K31/715

### B. FIELDS SEARCHED

IPC 7 A61L C12N

EPO-Internal, BIOSIS, PAJ, WPI Data

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 432 711 B1 (DINSMORE JONATHAN H ET AL) 13 August 2002 (2002-08-13)	1,2,8, 10-17, 22,23
Y	abstract column 5, line 62 - column 6, line 25; examples 1-3  ----- --/--	18-21, 24-26

☒ Patent family members are listed in annex.

**'&' document member of the same patent family**

12/08/2004

Mateo Rose11, A.M.



C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>SHIMIZU TATSUYA ET AL: "Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces." CIRCULATION RESEARCH. 22 FEB 2002, vol. 90, no. 3, 22 February 2002 (2002-02-22), pages e40-E48, XP002285777 ISSN: 1524-4571 cited in the application the whole document</p>	18-21, 24-26
Y	<p>TERUO OKANO: "Cardiac tissue reconstruction based on cell sheet engineering" JAPANESE CIRCULATION SOCIETY, 'Online! 2002, pages 1-4, XP002285778 Retrieved from the Internet: URL:http://www.j-circ.or.jp/english/sessions/reports/66th-ss/okano.htm&gt; 'retrieved on 2004-06-23! the whole document</p>	18-21, 24,26
X	<p>WO 01/07568 A (DIACRIN INC) 1 February 2001 (2001-02-01)  page 2, lines 18-24 page 3, columns 15-24 page 33, line 14 - page 34, line 7</p>	1,5,6, 9-17,22, 23
X	<p>KELLAR ROBERT S ET AL: "Scaffold-based three-dimensional human fibroblast culture provides a structural matrix that supports angiogenesis in infarcted heart tissue" CIRCULATION, vol. 104, no. 17, 23 October 2001 (2001-10-23), pages 2063-2068, XP002285779 ISSN: 0009-7322 abstract page 2064, left-hand column, paragraph 2 - page 2065, right-hand column, paragraph 1</p>	1,6,10, 16,17, 22,23

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MIYAGAWA SHIGERU ET AL: "A tissue engineered contractile cardiac graft improves the cardiac performance in infarct rat heart" 23 October 2001 (2001-10-23), CIRCULATION, VOL. 104, NR. 17 SUPPLEMENT, PAGE(S) II.599 , SCIENTIFIC SESSIONS 2001 OF THE AMERICAN HEART ASSOCIATION; ANAHEIM, CALIFORNIA, USA; NOVEMBER 11-14, 2001 , XP008031972 ISSN: 0009-7322 abstract	1, 10-15, 17-24, 26
P, A	BACH A D ET AL: "Expression of Trisk 51, agrin and nicotinic-acetylcholine receptor epsilon-subunit during muscle development in a novel three-dimensional muscle-neuronal co-culture system." CELL & TISSUE RESEARCH, vol. 314, no. 2, November 2003 (2003-11), pages 263-274, XP002285780 ISSN: 0302-766X abstract page 265, left-hand column, paragraph 4 - right-hand column, paragraph 1 page 266, right-hand column, paragraph 1 page 269, right-hand column, paragraph 1 - page 270, left-hand column, paragraph 1	1, 4, 5, 9-16

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 6432711	B1	13-08-2002	US 2002187550 A1	12-12-2002
			CA 2174746 A1	11-05-1995
			EP 0725817 A1	14-08-1996
			WO 9512665 A1	11-05-1995
WO 0107568	A	01-02-2001	AU 770889 B2	04-03-2004
			AU 6234600 A	13-02-2001
			CA 2378643 A1	01-02-2001
			EP 1200559 A2	02-05-2002
			JP 2003505475 T	12-02-2003
			WO 0107568 A2	01-02-2001
			US 2003113301 A1	19-06-2003
			US 6673604 B1	06-01-2004

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2004/001024

## Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
  - a. type of material
    - ☒ a sequence listing
    - ☐ table(s) related to the sequence listing
  - b. format of material
    - ☒ in written format
    - ☒ in computer readable form
  - c. time of filing/furnishing
    - ☒ contained in the international application as filed
    - ☐ filed together with the international application in computer readable form
    - ☐ furnished subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

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